Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 43. MARCH, 1940. NO. 3.

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11221 P

Relief of Hypochromic Anemia in Dogs with Synthetic Vitamin B_6: Influence of "Filtrate Factors."*

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Fouts, Helmer, Lepkovsky and Jukes[1] maintained dogs on a synthetic diet supplemented with all the known members of the vitamin B complex except B_6 (Factor 1, rat, anti-dermatitis vitamin), and

* Aided by a grant from the Christine Breon Fund for Medical Research.

1 Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., *J. Nutrition*, 1938, **16**, 197.

produced severe hypochromic microcytic anemia which was rapidly relieved with a rice-bran eluate rich in B_6. They later[2] reproduced the result with crystalline B_6 isolated from natural sources.[3] In the present report it is shown that synthetic B_6 (2-methyl, 3-hydroxy, 4,5-di-[hydroxy-methyl] pyridine)† will replace the natural material in the relief of this anemia.

Adult mongrel male dogs varying in weight from 7.5 to 21 kg were maintained on a purified diet of the following composition: casein (extracted for a week with acidulated water) 37%, sucrose 34.8%, cotton-seed oil 12%, lard 9%, cod-liver oil 3%, bone ash 2.4%, and salt mixture (Cowgill) 1.8%. The original salt mixture was modified by doubling the amount of ferric citrate. Supplements of various members of the vitamin B complex were given (per kg per day) as follows: thiamin chloride 50 μg, riboflavin‡ 50 μg, nicotinic acid‡ 1 mg, liver extract§ 0.06 ml. The liver extract was prepared from a crude liver concentrate by repeated treatment with fuller's earth to remove the adsorbable components. It was shown by rat assay to be rich in Factor 2 (rat growth factor) but practically free of B_6. Diet and water were given *ad lib.*

Four dogs, Nos. 74, 164, 165, and 188, maintained on this regime for from 120 to 135 days, developed hypochromic microcytic anemia with red blood cell counts of 5.78, 5.10, 4.95, and 4.91 million; hemoglobins of 6.3, 7.5, 7.2, and 5.9 g; mean corpuscular volumes of 51, 47, 49.5 and 45.8 cubic micra respectively. When the diet was started the red counts were 6.71, 6.87, 6.43, and 6.44 million; hemoglobins were 15.6, 14.7, 15.3, and 14.4 g; M C V were 64, 72, 75, and 64 cubic micra. Dog 74 was then given natural B_6 (obtained from Dr. Samuel Lepkovsky) in doses of 60 μg per kg per day. A slight reticulocytosis occurred (4% on the fifth day) and on the thirtieth day the red count was 7.0 million and the hemoglobin 11.6g. Dog 164 was given synthetic B_6 in doses of 60 μg per kg per day. The reticulocytes increased on the second day and reached a peak of 6.8% in 6 days. In 14 days the red count had risen to 5.5 million, the hemoglobin to 10.2 g, and the M C V to 61.8 cubic micra. On the thirty-fifth day the red count was 6.19 million, hemoglobin 12.6 g and M C V 66.3 cubic micra. Dog 165 was given 60 μg of synthetic

† Supplied by Dr. D. F. Robertson, Merck and Co., Rahway, N.J.

[2] Fouts, P. J., Helmer, O. M., and Lepkovsky, S., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 4.

[3] Lepkovsky, S., *J. Biol. Chem.*, 1938, **124**, 125.

‡ Supplied by the Galen Co., Berkeley, Calif.

§ Made and assayed by the Galen Co., Berkeley, Calif., and the Vitab Corp., Emeryville, Calif.

B_6 per kg per day. After a slight increase in reticulocytes to 4.6%, the red count and hemoglobin increased by the fourteenth day to 5.78 million and 9.5 g respectively. The M C V was 60.5 cubic micra. On the thirty-fifth day the red count was 6.24 million, hemoglobin 11.4 g and M C V 61.3 cubic micra. Dog 188 was given 60 μg synthetic B_6 per kg per day and in 14 days the red count was 6.1 million and the hemoglobin 9.5 g.

A control animal was maintained on the same regime with the addition of vitamin B_6, first in the form of a rice-bran eluate§ (free of the "filtrate factors") and later as synthetic B_6 in the amount of 60 μg per kg per day. He did not develop hypochromic anemia, but after several months he developed very mild normochromic normocytic anemia. This was found to be the result of a mild deficiency of the liver extract supplying the non-adsorbable "filtrate factors." Two animals maintained on the diet without addition of the filtrate but with 60 μg per kg per day of B_6 developed moderate anemia of the normochromic normocytic type.

At the start of the experiment the requirement of the dog for the non-adsorbable factors other than nicotinic acid was not known and we later found that the amounts given were insufficient. This prevented the complete relief of the hypochromic anemia by the administration of B_6, as the following data show. Dog 74, as noted above, responded rapidly to crystalline B_6. After the thirty-first day of treatment, however, the hemoglobin failed to rise further and during the next 14 weeks it remained at about the same level, fluctuating between 10.7 and 11.6 g. The red count slowly dropped from 7.0 to 5.4 million. Substitution of synthetic for natural B_6 in both the same and double dosage had no effect. Addition of rice-bran eluate (containing only adsorbable factors) and increase of riboflavin had no effect. When finally the liver filtrate level was tripled, the hemoglobin rose within 28 days to 13.9 g, the red count to 6.28 million and the M C V from 51.5 to 73. An adequate supply of a factor (or factors) present in the filtrate after exhaustive adsorption with fuller's earth is apparently necessary for complete relief of the anemia resulting from deficiency of B_6. This interrelationship is being further investigated. The filtrate used is known to be a rich source of the chick anti-dermatitis factor and the rat growth vitamin, but it cannot be said that the factor required by the dog is identical with either of these.

In addition to the anemia, the dogs deficient in B_6 presented other manifestations. All except one lost weight and all exhibited marked listlessness and asthenia. The lassitude was not entirely the result of the anemia, since it improved remarkably after only a few days

of treatment with B_6 before the hemoglobin had appreciably increased.

Conclusions. Synthetic vitamin B_6 relieves the hypochromic microcytic anemia produced in dogs deficient in this factor. An adequate supply of the non-adsorbable fraction of the vitamin B complex is necessary for the complete disappearance of this anemia.

11222 P

Response of Hypophysectomized Rats to Highly Purified Extracts of Pregnant Mare Serum.

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Goss and Cole[1] have shown that extracts of mare serum can be prepared testing 4000 to 7000 R.U. per mg total solids (40,000 to 70,000 R.U. per mg nitrogen). The question arises regarding the nature of the hormone present in these extracts as compared to that in untreated mare serum. Evans, *et al.*,[2] and Hellbaum[3] have reported that more than one gonadotropic hormone is present in mare serum though these reports have not been confirmed. In the first mentioned paper the male rats showing only a Leydig tissue response were possibly sacrificed too soon to expect an effect upon the seminiferous epithelium. Further, had the time of autopsy of the females been delayed beyond 72 hours corpora lutea might have been encountered following the treatment with so-called FSH fractions. The extracts prepared by these authors were relatively crude preparations. We were interested, therefore, in determining whether or not highly purified materials would give similar evidence of fractionation of gonadotropic hormones. A fraction giving pure follicular stimulation in the female with little or no effect on the interstitial tissue of the male would give evidence of a purified follicle stimulating hormone whereas a converse relationship would indicate the presence of a luteinizing hormone.

Experimental. Two extracts were used: one, No. 15-19-2, tested

1 Goss, H., and Cole, H. H., *Endocrinology*, 1940, **26**, 244.

2 Evans, H. M., Korpi, K., Simpson, M. E., and Pencharz, R. I., *Univ. of California Publication in Anatomy*, 1936, **1**, 275.

3 Hellbaum, A. A., *Am. J. Physiol.*, 1937, **119**, 331.

TABLE I.
Results on Males Injected with 20 R.U. of No. 15-19-2 Daily for 20 Days.

	Hypophysectomized		Treatment		Autopsy			
Rat	Age in days	B.W. (g)	Age in days	Daily dose, rat units	Age in days	Testes (mg)	Seminal vesicles (mg)	Prostate (mg)
G6953-C	40	112	75	20	96	660	440	418
W6952-C	40	118	75	20	96	681	433	412
BH6941-C	40	114	75	20	96	597	397	351
B6950-C	40	112	None; hyp'd control Uninjected		96	240	9	25
B-6926-C	Normal unoperated	123	0		40	1250	20	90

35,000 and the other, No. 17-76-4, 40,000 R.U. per mg nitrogen.* Four to 6 female rats, hypophysectomized on the 23rd day of age, were used at a given level for each extract. The intraperitoneal administration of a total of 30 R.U. over a 3-day period beginning on the 30th day of age produced ovaries averaging 116 mg for No. 15-19-2 and 127 mg for No. 17-76-4, on the 5th day after the initial injection. Sixty rat units produced ovaries weighing 122 mg and 101 mg, respectively. Estrous smears were encountered in all test animals. The macroscopic observation of many ripe follicles with some corpora lutea was confirmed microscopically.

The extracts were also tested in hypophysectomized male rats. The results on one extract, No. 15-19-2, are shown in Table I. There was a strong interstitial cell response as is indicated by the response of the accessory organs. Two rats treated similarly with No. 17-76-4 gave like results.

Thus it is seen that these extracts containing much less inert material than any previously reported for mare serum give a good follicular response in the hypophysectomized female and a strong interstitial cell response in the hypophysectomized male. In other words, in spite of the high degree of purification attained in the present extracts, no evidence was secured to support the view that mare serum contains 2 distinct hormones, one specifically affecting the interstitial tissue of the ovary and the Leydig tissue of the testis, and a second causing follicular growth in the female and germinal tissue development in the male.

* The potency of the extracts was determined in normal immature rats according to the method of Cole, Guilbert and Goss.[4]

[4] Cole, H. H., Guilbert, H. R., and Goss, H., *Am. J. Physiol.*, 1932, **102**, 227.

11223

Quantitative Response of the Dysentery Bacillus to Nicotinamide and Related Compounds.*

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In a previous report we showed that many strains of dysentery bacilli require nicotinamide or certain closely related compounds for growth.[1] We have also studied the growth-promoting activity of a number of compounds structurally related to nicotinamide.[2, 3] It is the purpose of this paper to report certain quantitative studies on the activity of nicotinic acid, nicotinamide, coenzyme I and coenzyme II. These studies were carried out in an attempt to learn the mechanism of action of these compounds in promoting growth of dysentery bacilli. The results were also used for the development of a method for the estimation of nicotinamide and related compounds in blood.

It has been generally assumed that nicotinic acid is converted to nicotinamide and that the latter is converted to one or both of the pyridine-containing coenzymes. We have presented evidence for the conversion of the acid to the amide, based on the relative activity of the two substances in promoting growth of dysentery bacilli.[3] It was found that when the acid is used as a growth-promoting substance there is a very definite lag in growth as compared with that obtained when the amide is used.

If nicotinamide is converted to one of the known pyridine-nucleotides, one or both of them must have a growth-promoting activity greater than or equal to that of an equivalent amount of nicotinamide.

In order to determine the relative activities of the various substances the titration method previously described by us was employed.†[3] Figure 1 indicates the relative activity of nicotinamide,

* This work was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago and a grant from the Committee on Scientific Research of the American Medical Association.

1 Koser, S. A., Dorfman, A., and Saunders, F., Proc. Soc. Exp. Biol. and Med., 1937, **38**, 311.

2 Dorfman, A., Koser, S. A., and Saunders, F., *J. Am. Chem. Soc.*, 1938, **60**, 2004.

3 Dorfman, A., Koser, S. A., Reames, H. R., Swingle, K. F., and Saunders, F., *J. Inf. Dis.*, 1939, **65**, 163.

† We are indebted to Professor Otto Warburg for a sample of pure coenzyme II and to Dr. A. Axelrod for a sample of pure coenzyme I prepared in Euler's laboratory.

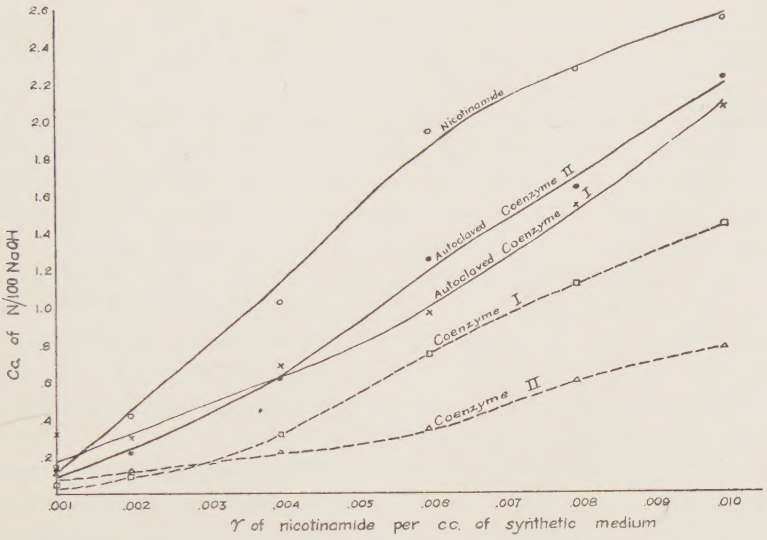

FIG. 1.

coenzyme I, coenzyme II, and autoclaved coenzymes I and II. It will be noted that nicotinamide is the most active growth-promoting substance, and coenzyme I is somewhat more active than coenzyme II. After the coenzymes are autoclaved (20 pounds, 30 minutes) the activity of each is markedly increased. Under certain conditions of heating it is possible to bring the activity up to that of an equivalent amount of nicotinamide. The results discussed above are obtained when the culture is titrated at the end of 4 days' incubation. If a shorter period is used the results are more striking since the coenzymes exhibit a lag period similar to that reported by us for the acid.

These findings are incompatible with the theory that nicotinamide functions solely as a building block for coenzyme I or coenzyme II. If nicotinamide is more active than an equivalent amount of either of the pyridine-containing coenzymes, it must have some importance other than, or in addition to, being a component of the known coenzymes. Metabolic experiments have confirmed this hypothesis although the details of this new mechanism are not yet known.[4]

Because of the rapid accumulation of evidence for the great importance of nicotinamide and related compounds in the nutrition and metabolism of various living forms there have been numerous attempts to develop methods for the determination of these compounds in biological materials.

[4] Dorfman, A., Koser, S. A., and Saunders, F., *Science*, 1939, **90**, 544.

The method which we have used is essentially the same as that described by us in a previous publication.[3] It consists in determining the amount of acid produced by a dysentery culture to which a definite dilution of a blood filtrate is added. The nicotinamide equivalent is determined by comparison with a standard curve (Fig. 1). The dilutions of the blood sample were made so that a minimum of 0.2 cc and a maximum of 0.4 cc was added to each tube containing 4.5 cc of basal medium. The final volume was made up to 5.0 cc. The tubes were incubated for 4 days at 37° and were then titrated with 0.01 *N* NaOH to a standard color of brom thymol blue. All blood samples were run at 2 different dilutions. Blank determinations were made on both inoculated and uninoculated controls. A series of nicotinamide standards was run with each set of blood samples. All determinations were run in triplicate.

The addition of a mixture of all of the available other known growth substances resulted in no increase in acidity, thus indicating that this test is specific for nicotinamide and related substances.[3] Schmelkes[5] has reported that thiazole-5-carboxylic acid is able to substitute for nicotinamide in promoting growth of dysentery bacilli. We have found that the activity of this compound is approximately one-hundred-thousandth that of nicotinamide, and therefore even if it did occur naturally it would not interfere with this assay.[6]

We first attempted to prepare our samples for assay by means of an acetone extract. It was found, however, that blood samples prepared in this manner gave results which were lower than those obtained with a water extract. Euler[7] has used an acetone method to determine the amount of free nicotinamide in blood. He obtained values of about 1 γ per cc when he used the cyanogen bromide method for determination of the nicotinamide. Our values when we used acetone for the preparation of the samples were somewhat higher, the normals averaging about 3 γ per cc. This difference is probably due to the fact that we employed 3 volumes of acetone, while Euler used 10 volumes. The latter quantity would extract less of the coenzymes.

The method used in most of our studies consisted in adding one volume of whole blood to 3 volumes of water within 10 minutes after the samples were drawn. This mixture was then heated to 70° for about 10 minutes and the coagulum filtered off. The preliminary heating is necessary to destroy the enzymes in blood which hydrolyze the coenzymes. The supernatant liquid is then ready for assay.

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5 Schmelkes, F. C., *Science*, 1939, **90**, 113.

6 Koser, S. A., Dorfman, A., and Saunders, F., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 391.

7 Euler, H. V., and Schlenk, F., *Klin. Woch.*, 1939, **18**, 1109.

TABLE I.
Effect of Autoclaving on Amount of Nicotinamide Found in a Sample of Human Blood.

Determination No.	Dilution of filtrate, cc	N. amide added per cc, γ	Method of sterilization	Value found, γ per cc
1	.020	—	Filtered	6.1
	.030			6.2
2	.025	5	,,	10.3
	.015			11.2
3	.025	—	Autoclaved	7.5
	.015			8.4
4	.015	5	,,	13.2
	.010			12.0

Table I illustrates the results obtained on a normal sample of blood. The value obtained for total activity expressed as nicotinamide is dependent upon the method of sterilization employed. In this experiment the same blood was employed for all analyses. It will be noted that the activity is markedly increased by heat treatment. More drastic treatment resulted in little increase in activity. This increase in activity is to be expected on the basis of the results reported in the first part of this paper, since it is known that most of the nicotinamide present in blood exists in the combined form.

Table I also illustrates the type of results that can be obtained by running the samples at different dilutions, and the recovery of nicotinamide when added to the whole blood. We have occasionally experienced difficulties in obtaining checks at different dilutions. The explanation for these difficulties was not found.

Kohn[8] has pointed out that the amount of "V" substance present in human blood is a function of the hematocrit. In an earlier report we showed that all of the activity for the dysentery bacilli resides in the erythrocytes.[9] Table II shows the results obtained with 5 different bloods both before and after autoclaving, together with hematocrit values. The number of samples analyzed by us is insufficient to permit drawing any definite conclusions concerning the relation between hematocrit and nicotinamide.

The results given in Tables I and II are typical for normal human samples.

Summary. By means of a titration method for estimation of bacterial growth it was found that growth is proportional to the quantity of nicotinamide present. Nicotinamide is more active than an

8 Kohn, H. I., and Bernheim, F., *J. Clin. Invest.*, 1939, **18**, 585.

9 Dorfman, A., Horwitt, M. K., Koser, S. A., and Saunders, F., *J. Biol. Chem.*, 1939, **128**, xx.

TABLE II.
Nicotinamide Content of Human Blood.

Sample	Dilution of filtrate	Method of sterilization	Value found, γ/cc whole blood	Hematocrit
A	.03	Filtered	.60	42.0
	.02		.54	
A	.03	Autoclaved	.80	42.0
	.02		.70	
B	.03	Filtered	.58	46.5
	.02		.55	
B	.03	Autoclaved	.88	46.5
	.02		.92	
C	.03	Filtered	.64	53.0
	.02		.72	
C	.03	Autoclaved	.91	53.0
	.02		.97	
D	.03	Filtered	.45	41.0
	.02		.52	
D	.03	Autoclaved	.83	41.0
	.02		.85	
E	.03	Filtered	.58	46.0
E	.03	Autoclaved	.75	46.0

equivalent amount of either pyridine-containing coenzyme. Hydrolysis increases the activity of the latter, indicating that the function of nicotinamide is not based entirely on synthesis to either of the known coenzymes. A method has been developed for determining nicotinamide and related substances in blood. The values obtained are higher if autoclaved blood is used.

11224

Rapidity of Passage of Chloride Ion from Blood into Gastric Juice of Stimulated Stomach.*

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In order to study the passage of chloride from the blood into the acid gastric juice of the stimulated stomach, chloride ions were "tagged" by rendering them radioactive. Thus radioactivity detected in the juice would signify that such ions if previously injected into the blood had been brought through the gastric mucosa.

* These observations are incidental to a study of achlorhydria in gastric carcinoma supported by grants from the National Advisory Council on Cancer, Washington, D.C., and The International Cancer Research Foundation, Philadelphia, Pennsylvania.

† Research Assistant, International Cancer Research Foundation Grant.

Radio-chloride has a half life of approximately 35 minutes and lithium a half life of 1/25 second. Thus lithium chloride was employed as a target for bombardment by the beam in the cyclotron of the Departments of Chemistry and Physics. Because of the brief radioactivity of the lithium the salt afforded a soluble chloride in which only the latter ion was radioactive. The lithium chloride was dissolved in sterile water and a drop or two of 1/10 N NaOH added to insure slight alkalinity. The solution was boiled gently for sterilization and then injected intravenously at the desired moment. The injections were made within 45 minutes after the material was received from the cyclotron and the observations completed within 3 hours. Radioactivity was determined by holding approximately 1 to 3 cc of the fluid to be tested in cellophane sacs 1 to 1.5 cm from the aluminum foil-covered aperture of a quartz-fiber Lauritsen electroscope. Proper control observations were of course made upon the "background" of the room in which the readings were made and upon blood, urine and gastric juice samples before injection of the radiochloride.

Experiment I. Female dog An. Wt. 10 kg with large cannulated gastric pouch. After several feedings of cooked lean meat vigorous pouch secretion developed and was collected every 10 minutes in Soxhlet flasks suspended below the cannula. These samples varied from 1 to 1.75 cc per 10-minute period with free acid varying between 100 and 150 clinical units and combined acid 20 to 30 clinical units. At the termination of one period 500 mg of radioactive lithium chloride dissolved in 10 cc of water were injected intravenously in a hind extremity. The volume of juice secreted during the next 10-minute period equalled 3 cc and when tested was found to be strongly radioactive. Similar samples collected at 10-minute intervals for the next 130 minutes each showed radioactivity.

Experiment II. Male Dog Gyp. Wt. 10 kg with large cannulated gastric pouch. Procedure as in Experiment I. In this case 3 cc were secreted during the 120 seconds following the termination of the injection of lithium chloride. This sample was tested separately and found to be strongly radioactive. Tests were also made on blood and urine samples, the results are indicated in the accompanying graph.

Experiment III. Patient, Mrs. H., age 58, Wt. 50 kg, thin and emaciated, 8 days postoperative cholecystectomy for cholecystitis and cholelithiasis. A Levine tube was passed into the stomach after 18 hours' starvation and gastric lavage performed with water, all of the latter being withdrawn. One milligram of histamine was injected subcutaneously and 10 minutes later gastric aspirations were begun,

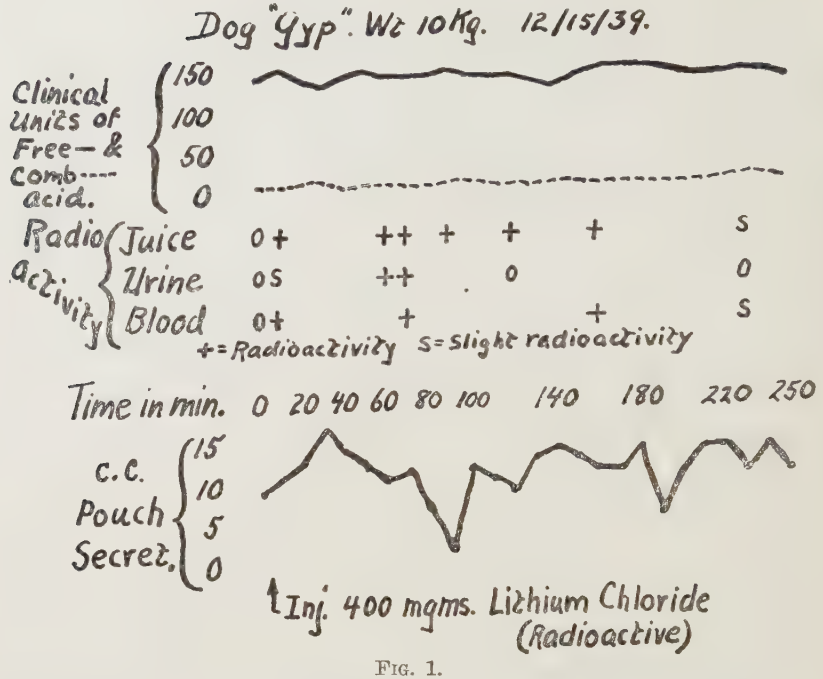

FIG. 1.

Graph of Exp. II described in text showing appearance time of radioactive chlorine in pouch gastric juice subsequent to its injection into an extremity vein. Persistence of the "tagged" ions in the blood and their presence in the urine are also indicated.

and continued at similar intervals for 90 minutes. Following the third aspiration which yielded a juice containing 25 clinical units of free acid and combined acid of 12 clinical units, 400 mg of radioactive lithium chloride dissolved in 10 cc of water were injected intravenously in the arm. During the 120 seconds following the termination of this injection 1½ cc of gastric juice were aspirated which showed radioactivity. Six subsequent samples aspirated at 10-minute intervals all showed radioactivity. Five cc of blood withdrawn from the anticubital vein 1 minute and 70 minutes respectively following injection showed radioactivity. Prior to the experiment the patient voided. At the termination of the experiment the patient voided 36 cc. Three cc of this urine showed radioactivity but approximately one-half as intense as the last sample of 3 cc of gastric juice tested.

Experiment IV. Patient, St., white male, 23, one week after bilateral herniorrhaphy. Procedure as in Exp. III. 3 cc of gastric juice aspirated 60 to 90 seconds following the termination of the injection of radio chloride showed radio-activity. The free acid in this sample equaled 110 clinical units, the combined acid, 17 clinical units.

As a control for the method, radioactive phosphorus in the form of sodium phosphate was injected intravenously into three pouched dogs. The blood was observed to be radioactive for over 45 minutes but the gastric pouch juices secreted during this period exhibited no radioactivity, according to the criteria adopted in these experiments.

Summary. The foregoing observations show that "tagged" chloride ions when injected into the general circulation are identified in the acid gastric juice of the stimulated stomach in dog and in man as quickly as 60 to 120 seconds after injection. Presumably at least some of these ions in the juice were derived from the HCl present. Tagged chloride ions continued to circulate in the blood for more than one hour after intravenous injection and were transported into the gastric juice continuously during this period. Such ions did appear in the urine but were detected at irregular intervals and in relatively low concentration.

11225

Availability of Staphylococcal Antitoxin After Intramuscular Injection into Normal Monkeys and Men.*

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The increasing use of staphylococcal antitoxin[1, 2] makes it advisable to study its rate of absorption and concentration in the blood after injection of therapeutic doses. In accordance with previous work on diphtheric antitoxin[3] and antipneumococcal serums,[4] daily estimations were made of the titers of 6 "normal" human subjects, who were not suffering from staphylococcal infections and of 9 normal monkeys *(M. mulatta)* after intramuscular injections of Squibb's concentrated antitoxin. Some of each group were injected into the gluteal muscle with 1000 units per kg, while others received 2 such doses, 24 hours apart (Table I). *Alpha* antistaphylolytic serum

* Aided by a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, of the American Medical Association.

1 Baker, L. D., and Shands, A. R., *J. A. M. A.*, 1939, **113**, 2119.

2 Stookey, P. F., and Scarpellino, L. A., *So. Med. J.*, 1939, **32**, 173.

3 Glenny, A. T., and Hopkins, B. E., *J. Hygiene*, 1924, **22**, 12-36; 208-222.

4 Finland, M., and Brown, J. W., *J. Immunol.*, 1938, **35**, 245.

TABLE I.

Summary of Data on Normal Monkeys and Persons Injected Intramuscularly with Varying Doses of Concentrated Staphylococcal Antitoxin (Squibb) and Rabbits* Injected Intravenously with Diphtheric Antitoxin.

Animal or patient No.	Wt, kg	Plasma volume, cc	Units of antitoxin per kg	Max. increments of serum titers (Units *alpha* antistaphylolysin per cc)	Sums of conc. of antibody 9 days (Areas)	Avg of areas	Antibody available in total plasma vol.	Avg
				Normal monkeys (*M. mulatta*).				
229	3.0	162.0	1000	25	110	113.5	17,800	19,160
253	3.4	183.6	1000	26	131		24,050	
243	2.5	135.0	1000	14	86		11,600	
244	2.75	148.5	1000	29	137		20,340	
252	4.0	216.0	1000	19	103		22,000	
254	2.7	145.8	2000	35	170	233.8	24,800	32,620
230	2.6	140.0	2000	45	278		39,030	
255	2.0	108.0	2000	38	270		29,160	
251	3.2	172.8	2000	38	217		37,500	
				"Normal" human subjects.				
1. M.R. (g)	57	3,078	1000	8.5	48	43	147,700	141,550
2. R.G. (a)	66	3,564	900	6	38		135,400	
3. N.C. (c)	56	3,024	2100	15	101	93	305,420	288,115
4. J.F. (m)	59	3,186	2000	25	85		270,810	
				Normal rabbits* (injected intravenously with 750 units diphtheric antitoxin.				
83 f	3.24	129.6	231.0	5.25	12.03		1,558	
109 f	2.84	113.6	264.0	7	15.90		1,806	
110 f	2.81	112.4	267.0	8	15.07		1,694	
94 y	0.91	36.4	824.0	29	34.17		1,244	
84 y	0.40	16.0	187.0	16	79.24		1,268	

(g) gastric ulcer; male, age 59.
(a) arthritis; male, age 31.
(c) non-specific conjunctivitis; male, age 25.
(m) moron; male, age 17.
*Illustrative data selected from tables of Glenny and Hopkins.[3]
y very young animals.
f full grown; almost mature rabbits.

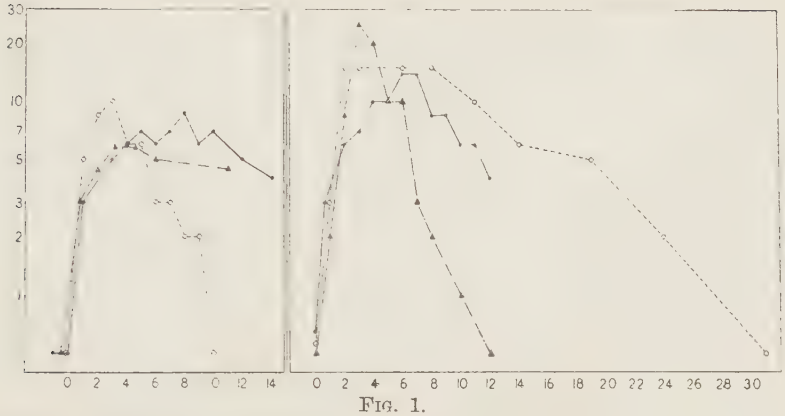

FIG. 1.

Rate of absorption of antistaphylococcal antitoxin after intramuscular injection into "normal" human subjects. Left graphs, 3 persons injected with 1000 units per kg; right graphs, 3 injected with 2000 units per kg. Ordinates: logarithms of units of *alpha*-antistaphylolysin per cc of serum; abscissæ, days after injection.

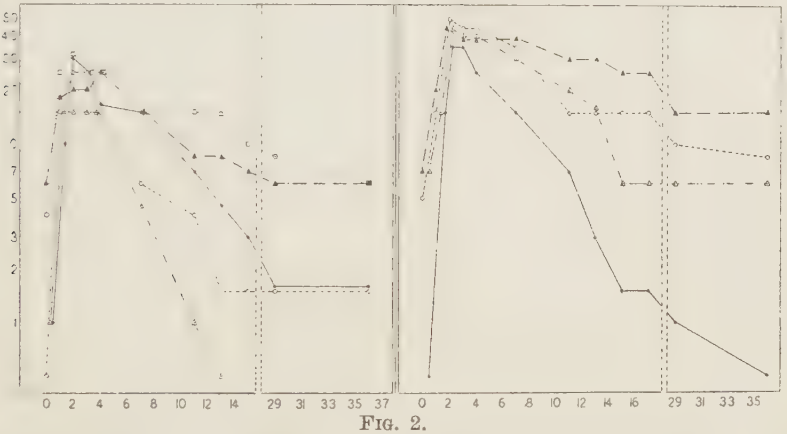

FIG. 2.

Rate of absorption of antistaphylococcal antitoxin after intramuscular injection of normal monkeys (*M. mulatta*). Left graphs, 5 monkeys injected with 1000 units per kg. Right graphs, 4 monkeys injected with 2000 units per kg. Coördinates as in Fig. 1.

titers were determined by the method of the National Institute of Health.[5]

Figs. 1 and 2 show striking uniformity in the rates of absorption of *alpha*-antistaphylolysin. Semi-logarithmic curves present 3 phases. In humans, phase A, the stage of absorption, lasted 3 to 8 days. Phase B, the period of maintenance of maximal serum titers, 1 to 6 days, the average being 3 days. Phase C, the stage of elimination of antibody.

5 National Inst. Health, Washington, D.C., B-1199, Jan., 1936, U. S. Standard Staphylococcus Antitoxin.

extended from 2 to 4 weeks. The maximal increments in titer after 1000 units per kg of antitoxin, varied from 6 to 10 units. After 2000 units, maximal titers varied from 15 to 25 units. In monkeys injected with similar doses, phase A lasted 1 to 4 days; phase B, 3 to 6 days; phase C, from 4 to 5 weeks. The maximal increments were much higher than in humans—the titers ranging from 15 to 30 units after 1000 units of antitoxin and 35 to 50 after 2000 units per kg. It must be emphasized that the natural titers of monkeys are higher than those of humans. In the latter they were less than 1 unit whereas in monkeys they varied from less than 1 to 7 in this series, but may reach as high as 20 or more units per cc.[6]

In most instances, the serum titers reverted to their natural levels after elimination of the passively transferred antibody.

Because of individual variations in maximal serum titers and in the time of elimination of the antitoxin, it became necessary to develop a method of indicating the total available circulating units of antitoxin during a given period of time as the result of the injection of a given dose. By plotting on standard graph paper, concentrations of *alpha* antistaphylolysin per cc of serum as ordinates and time, in days after injection, as abscissæ, and integrating these curves, the areas underneath represent the summation of concentrations of antibody that have left their storehouse in the reticulo-endothelial system[7] and appeared in the blood stream during the period of observation. The areas may be conveniently determined by weighing the paper under the curve, counting the units squares or by use of a planimeter.

When 5 monkeys were each injected with 1000 units per kg, the average concentration of available antibody during 9 days was 113.5 units per cc (Table I). In 4 other monkeys similarly injected with twice the dose per kg, the average was double or 233.8. In human subjects the values for similar dosage and time-intervals were approximately 43 and 93 respectively, the ratio being again approximately 1:2. Multiplying these values by the weights and the factor 54, one obtains the number of units of antitoxin appearing in the total plasma-volume.[8] Thus, in persons receiving 1000 units per kg, the total available circulating units in the course of 9 days was 141,550, whereas in 2 others receiving approximately twice this dose per kg, the total was about twice as large, or 288,115. The corresponding average values for monkeys are 19,160 and 32,620. In all instances,

[6] Weiss, C., *J. Immunol.*, 1939, **37**, 185.

[7] Sabin, F. R., *J. Exp. Med.*, 1939, **70**, 67.

[8] Brown, G. E., and Keith, H. Z., quoted in Peters, J. P., and van Slyke, D. D., *Quantitative Clinical Chemistry*, Williams and Wilkins, Baltimore, 1935, **1**, 725.

therefore, irrespective of the maximal titers or the duration of elimination, the sum of the concentrations of antibody during a given period of time (area under the curve) or the antibody content of the total plasma volume was directly proportional to the dose injected per kg weight.

This new procedure was also applied to data selected from Glenny and Hopkins.[3] They injected a constant dose of diphtheric antitoxin (750 units) into each of 5 rabbits weighing from 0.400 to 3.240 kg. Although the maximal titers and duration of elimination varied, as reflected in the sums of the concentrations of antibody during 9 days (12 to 79), nevertheless the antibody available in the total plasma was practically identical in all animals. In other words, with a given total dose injected, the turnover of antibody in the plasma is the same.

Preliminary observations on *beta* antistaphylolysin[9] revealed a uniform rate of absorption but the serum titers reached were very small, never exceeding 1 to 6 units, in normal persons and monkeys respectively. This may be explained by the low concentrations in commercial antitoxin which did not exceed 15 and 80 units per cc in Lederle's and Squibb's antitoxin respectively.

Summary and Conclusions. After intramuscular injection of staphylococcal antitoxin the rate of absorption is quite uniform in normal monkeys and humans. The antibody available in the total plasma-volume during a given period of days after injection of a specified dose (per kg weight) may be determined from the area under the curve, formed with time intervals as abscissæ and daily concentrations of antibody per cc as ordinates, multiplied by the plasma volume. This new method of calculating available circulating antibody has also been applied successfully to the data of Glenny and Hopkins[3] on diphtheric antitoxin and hence may be of general immunological interest.

9 Smith, M. L., and Price, S. A., *J. Path. and Bact.*, 1938, **47**, 361.

11226 P

Serum Volume Index Studies on Newborn Children.

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(Introduced by Richard Ashman.)

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Although reports differ in detail,[1–5] it has been fully established that a hypoprothrombinemia occurs in infants shortly after birth, that its duration is brief, and that normal values are usually reëstablished by the fifth day of life. That this deficiency is related to hemorrhagic disease of the newborn is suggested by the fact that the hemorrhagic state coincides with the period of hypoprothrombinemia and by its prompt correction by vitamin K therapy.[3–6]

Previous studies have been made by the prothrombin titration technic of Quick,[7] the two-stage titration technic of Warner, Brinkhous and Smith,[8] the bedside test of Ziffren, Owen, Hoffman and Smith,[9] and the coagulation deficiency test of Dam and Glavind.[10] The studies herewith reported were made by the serum volume test,[11, 12] which was devised to predict the hemorrhagic diathesis in jaundice and has been used with entire satisfaction for this purpose for the last 5 years.

The studies were made on 6 white and 5 colored mothers and their full-term babies. The maternal blood was secured from the antecubital vein. The first samples in the children were secured from the

1 Brinkhous, K. M., Smith, H. P., and Warner, E. D., *Am. J. M. Sc.*, 1937, **193**, 475.

2 Quick, A. J., and Grossman, A. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 227

3 Hellman, L. M., and Shettles, L. B., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 138.

4 Waddell, W. W., Jr., and Guerry, DuP., *J. A. M. A.*, 1939, **112**, 2259.

5 Shettles, L. B., Delfs, E., and Hellman, L. M., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 419.

6 Dam, H., Tage-Hansen, E., and Plum, P., *Lancet*, 1939, **2**, 1157.

7 Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. M. Sc.*, 1935, **190**, 501.

8 Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

9 Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 595.

10 Dam, H., and Glavind, J., *Acta med. Scandinav.*, 1938, **96**, 108.

11 Boyce, F. F., and McFetridge, E. M., *J. Lab. and Clin. Med.*, 1937, **23**, 202.

12 Boyce, F. F., and McFetridge, E. M., *New Orleans M. and S. J.*, 1939, **91**, 357.

cord, and the second and third from the jugular vein or the anterior fontanel. The serum volume index is determined as follows:

Five cc of blood is allowed to stand at room temperature in a graduated tube for 4 hours, at the end of which time the serum volume expressed from the clot is read. A red blood cell count is also made. In normal individuals the serum expressed is 50% of the blood volume. The index equals the serum volume of the patient studied over half the volume of blood withdrawn for the test; a correction is made for anemia if it exists. One (1) is the standard of normal. The highest index at which a jaundiced patient has bled is .71.

The maternal values (Table I) in these 11 patients are rather lower than are normally found, the decreases perhaps being due to the loss of blood at delivery. Some confirmation of this supposition is furnished by the almost uniform rise in the second and third maternal indices. The infant indices parallel the observations made by other tests in all respects: They are considerably lower than the maternal indices, the lowered values found immediately after delivery are still further decreased on the third day of life, and there is a uniform rise, usually with a return to the initial values or higher values, on the fifth day of life. The values reported are paralleled by values in other, less complete, maternal and fetal studies by the serum volume index and also by values in studies by the bedside test[9] to be reported elsewhere.

In 4 instances the fetal indices immediately after birth fell within the range at which bleeding would be expected in the adult, and 9 fetal indices on the third day were well below the safe level. Bleeding did not occur in any of these children, nor does it seem to have occurred in other instances[1, 2] in which the prothrombin values were well

TABLE I.
Studies by the Serum Volume Index (for the Hemorrhagic Diathesis) in Parturient Women and Newborn Children.

Mother			Child		
At delivery	Third day	Fifth day	At delivery	Third day	Fifth day
.76	.67	.87	.76	.77	.82
.87	.91	.91	.70	.65	.64
.87	.88	.80	.86	.57	.73
.80	.86	.88	.71	.65	.72
.88	.94	.91	.66	.60	.63
.71	.80	.80	.65	.61	.81
.81	.94		.96	.47	
.75	.80	.81	.99	.88	.98
.83	.81	.80	.71	.62	.73
.87	.89	.92	.89	.51	.75
.89	.89	.92	.69	.61	.57

below normal. Whether children bleed at a lower level than adults, so that a fresh set of standards must be devised for them, it is not now possible to say. A partial explanation of the lower maternal and fetal values may be that all of these tests were run during the winter, when the intake of foodstuffs containing vitamin K is less than in the summer months.[5]

Summary. A serial study of 11 parturient women and their infants by the serum volume index confirmed the findings of others, by other tests, that a definite tendency to hemorrhage exists in infants for a short time shortly after birth.

11227 P

Fleas as Acceptable Intermediate Hosts of the Dog Heartworm, *Dirofilaria immitis.*

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The method of natural transmission of the dog heartworm, *Dirofilaria immitis,* is still incompletely elucidated. The larvae of this parasite have been shown to develop in many species of mosquitoes and, thus, mosquitoes have been believed to be incriminated in the natural transmission, but substantial proof of this is lacking. The possibility that other blood-sucking arthropods serve as transmitters was overlooked until 1921, when Breinl[1] reported finding the microfilarial embryos of *Dirofilaria immitis* in the fleas, *Ctenocephalides canis* and *Ctenocephalides felis,* collected from heartworm infected dogs in Australia. He also observed the advanced stages of development of this parasite in the Malpighian tubules of the fleas and the infective stage larvae in the hemocelic cavity. Recently, Brown[2] has observed the microfilariae and partially developed larvae of this parasite in *Ctenocephalides canis* collected from heartworm-infected dogs in the eastern United States.

In November of 1938, the writer, in examining dogs for heartworm infection in New Orleans, observed the typical microfilariae of *Dirofilaria immitis* in *Ctenocephalides canis.* The early developmental stages and the infective larvae were also seen in the hemocelic

1 Breinl, A., *Ann. Trop. Med.,* 1921, **14**, 389.

2 Brown, H. W., *The North Amer. Veterinarian,* 1939, **20**, No. 1.

cavity of fleas collected from a dog having a heavy infection. On other dogs harboring *Dirofilaria immitis Ctenocephalides felis* and *Pulex irritans* were found similarly infected. The female fleas of the 3 species were much more frequently infected and were more heavily infected with all the larval stages of the parasite than were the males. From some dogs infected with heartworm as many as 100% of the female fleas examined contained one or more infective-stage larvae and many of the earlier stages of development. The males harbored these stages only in small numbers and rarely contained the infective stage larvae.

Fleas of these 3 species collected from uninfected dogs have been experimentally infected in the laboratory in New Orleans and the extra-mammalian phase of the life cycle of *Dirofilaria immitis* has been completed in 10 such experiments undertaken. Sections of the infected fleas showed that the parasite, in the typical microfilarial stage as found in the dog's blood, had entered the ventral part of the hemocelic cavity in the region occupied by the fat bodies and had there begun its development. The metamorphosis of the parasite from the microfilaria to the infective stage larva followed closely that found in various species of mosquitoes infected here[3, 4] and by other workers elsewhere. The microfilaria developed into a shortened, so-called sausage larva, identical with that found in experimentally infected mosquitoes. By gradual elongation of the body and development of the internal structures this sausage larva became the infective stage larva, which was morphologically identical with that developing in mosquitoes, although the time required for complete development in the flea was much shorter than in any mosquito worked with here or as yet reported in the literature. Thus far the minimum time required for development in the flea, from the microfilarial to infective stage, was 120 hours as compared with 240 hours in species of mosquitoes experimentally infected here. In addition, the writer's observations indicate that the microfilaria did not begin its development in the Malpighian tubules, as Breinl had stated for fleas and as occurs in mosquitoes, but rather in the hemocelic cavity.

In experiments conducted with fleas during the months of June and July it was found that the infective stage larva of *Dirofilaria immitis* appeared in 120 hours as compared with 216 hours in the same species of flea in experiments conducted in December and January. Observations as yet incomplete seem to indicate that during

[3] Hinman, E. H., *Am. J. Trop. Med.*, 1934, **15**, 371.

[4] Writer's observations.

the warmer season of the year the time required for development is shorter than during the colder season. With decreasing temperature the time required for development increases and *vice versa.*

In summary, the fleas, *Ctenocephalides canis, C. felis* and *Pulex irritans,* collected from dogs harboring *Dirofilaria immitis,* have been found naturally infected with the microfilarial and larval stages of this parasite. The female fleas seemed much more susceptible to the infection than the males. All 3 species of fleas have been experimentally infected and in all 3 the extra-mammalian phase of the life cycle has been completed. The time required for this development in fleas was 120 hours in warm weather and 216 hours in cold weather. This phase of the life cycle was more rapidly achieved in fleas than in mosquitoes. It appears that both biologically and epidemiologically fleas are more suitable intermediate hosts of *Dirofilaria immitis* than had been previously supposed.

11228 P

Hyperthyroidism and Liver Function in Relation to B Vitamins.

VICTOR A. DRILL AND HARRY W. HAYS. (Introduced by W. W. Swingle.)

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Many authors have reported that the liver becomes altered both structurally[1] and functionally[2] during hyperthyroidism. One of the most marked changes in the liver during the experimental and clinical hyperthyroid states is a depletion of the liver glycogen.[1, 3] A high carbohydrate diet is usually given to hyperthyroid patients in an effort to promote glycogen storage in the liver.

Abelin[4] and Abelin, *et al.*,[5] showed that large amounts of casein, egg yolk, or yeast would lessen the liver damage of the thyroid-fed animals, and Drill[6] was able to prevent a fall in the liver glycogen of

1 *cf.* Frazier, C. H., and Brown, R. B., *Trans. Am. Assn. for Study of Goiter,* 1935.

2 Maddock, W. G., Pedersen, S., and Coller, F. A., *J. Am. Med. Assn.,* 1937, **109**, 2130. *cf.* reference1.

3 John, H. J., *J. Am. Med. Assn.,* 1932, **99**, 620.

4 Abelin, I., *Biochem. Z.,* 1930, **228**, 165.

5 Abelin, I., Knochel, M., and Spichtin, W., *Biochem. Z.,* 1930, **228**, 189.

6 Drill, V. A., *J. Nutrition,* 1937, **14**, 355.

thyroid-fed rats by feeding large amounts of yeast. It is also known that the dietary requirements for vitamin B_1 and the B_2 complex are above normal during experimental hyperthyroidism in rats.[7] Since yeast can maintain the liver glycogen of thyroid-fed rats we decided to investigate any possible relationships between the liver function of hyperthyroid dogs and the yeast content of the diet. No studies of the liver function of hyperthyroid animals are reported in the literature.

Methods. Throughout the experiment the dogs received a modified form of Cowgill's casein diet No. III,[8] in which 21% lard was used, and the butter was replaced by 4% of cod liver oil, so that a known amount of vitamins A and D were added to the diet. The dogs used were all full-grown males weighing between 9 and 17 kg. The dogs were fed a stock diet of Purina checkers *ad libitum*. Two weeks before the thyroid feeding was started the dogs were placed on the modified Cowgill diet. The dogs were allowed to eat as much as they wanted for a 3-hour period each day. Each dog received a daily supplement of yeast No. 17800 in the proportion of 2 International units of vitamin B_1 per pound of body weight. The yeast contained 23 I.U. of vitamin B_1 per gram and 20 Sherman-Borquin units of vitamin G (flavin) per gram.* This makes the diet normal in all respects. The thyroid used was Lilly's desiccated thyroid gland, U.S.P.

The bromsulphalein method[9] was used to determine the liver function of the dogs. Five mg of dye per kg of body weight was injected intravenously into the jugular vein of unanesthetized trained dogs, and 30 minutes later 10 cc of blood was withdrawn under oil from the opposite jugular and the concentration of dye remaining in the serum determined. In the standards 4 mg of bromsulphalein per 100 cc of dilute NaOH was used as 100%.

Results. The 2 control dogs, receiving only Cowgill's diet and the daily yeast supplement, showed normal liver functions throughout the experiment. The dye retention in the serum of the controls at the end of 30 minutes was always less than 10%, generally being between 2 and 8%. Any retention of dye above 15% in the thyroid-fed animals was therefore considered abnormal and definite evidence of impaired liver function.

7 Drill, V. A., and Sherwood, C. R., *Am. J. Physiol.*, 1938, **124**, 683.

8 Cowgill, G. R., *Am. J. Physiol.*, 1928, **85**, 45.

* The authors wish to thank Dr. H. W. Rhodehamel of Eli Lilly and Co. for supplying the large amount of desiccated thyroid gland that was required, and also Dr. C. N. Frey of the Fleischmann Laboratories for supplying the analysed yeast.

9 Rosenthal, S. M., and White, E. C., *J. Am. Med. Assn.*, 1925, **84**, 1112.

Dogs fed 0.6 gm thyroid gland/kg of body weight. Two dogs (Nos. 3 and 11) were fed this dosage. Dog No. 3 showed a normal liver function up to the 42nd day of thyroid feeding. On the 42nd day the yeast was reduced to one-half of its amount in the diet and 24 hours later the liver function showed 25% of dye retention. On the 44th day the yeast was removed from the diet and 24 hours later the test showed 150% retention of dye. More than 100% retention of dye is due to the fact that the colorimeter standards developed originally for the injection of 2 mg of dye per kg of body weight, have been retained even though a 5 mg dose is used. It is difficult to differentiate the color with higher standards.[2] The dye retention then dropped and remained at an average of 50% during the next 10 days. Dog No. 11 showed a marked drop in appetite after the 37th day of thyroid feeding. This indicated that the vitamin stores, in particular vitamin B_1, of the body were lowered and that now more vitamins were being metabolized than were being supplied in the normal diet. A liver function on the 41st day gave 45% retention.

Dogs fed 0.4 g of thyroid gland/kg of body weight. Dogs Nos. 2, 9, and 10 received this dosage. Dog No. 2 gave normal liver functions up to the 49th day of thyroid feeding. A test on the 59th day showed 25% retention. The yeast was then removed from the diet and 24 hours later 125% retention of dye was obtained. This dropped and remained between 25-40% during the next 10 days. Dog No. 10 showed similar results. Dog No. 9 gained a considerable amount of weight after the experiment had started, so that on his new weight basis he was receiving less than 0.4 g of thyroid/kg of body weight. His food intake was still above normal, and was not dropping, indicating that no depletion of the body stores of vitamins had occurred. On the 57th day his liver function was still normal, and no fall in appetite was observed. The yeast was removed from the diet on the 57th day and 24 hours later only 20% of dye retention was observed, which remained at this low level during the next 5 days. This shows that without vitamin depletion, as shown by the high food intake, that only a slightly abnormal liver function was obtained when the yeast was removed.

Discussion. The results indicate a definite relationship between the yeast content of the diet and the liver function of thyroid-fed animals.

Some of the dogs maintained normal liver functions for 40-50 days on the above diet while receiving thyroid gland, and then showed a marked abnormal liver function as soon as the yeast was removed. In the others the liver function became abnormal, without removing the yeast from the diet, in about 40 to 50 days. These

results are best obtained with a dosage of 0.6 g of thyroid gland per kg of body weight. It is known that the amount of vitamin B_1 in rat tissues is reduced by thyroid feeding,[10, 11] and that the thyroid-fed rat also requires more of the vitamin B_2 complex than is supplied in a normal diet.[7] We have found this same dietary relationship to be present in the dog.[12] This indicates that the abnormal liver functions in the thyroid-fed dogs are related to an increased requirement for some of the B vitamins, with a probable loss in body stores, as judged by the loss of appetite. This will be reported later in greater detail. A subnormal amount of the B vitamins in the diet may be at least partially responsible for the abnormal liver function that is observed in human hyperthyroidism.

The results of these experiments do not mean that the B vitamins are the only factors related to the production of abnormal liver function in hyperthyroid animals. However, this is the first causal relationship to be established for the production of abnormal liver function in hyperthyroidism. It is not yet known if a large amount of yeast in the diet can maintain a normal liver function in thyroid-fed dogs over a long period of time. Further experiments are in progress.

Conclusions. 1. Using a standard diet, with yeast of a known vitamin content, the liver functions of dogs was studied at two levels of thyroid feeding. 2. The production of the abnormal liver function in hyperthyroid dogs bears a causal relationship to the yeast in the diet.

11229

X-radiation and Growth Substances as Affecting Plant Primordial Tissues.

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Since little attention has been given to the primordial regions of plants as influenced by X-radiation and chemical growth-promoting substances the author has carried out experiments to determine effects of these agencies on growing points. Snow[1] observed that hetero-

10 Drill, V. A., *Am. J. Physiol.*, 1938, **122**, 486.

11 Peters, R. A., and Rossiter, R. J., *Biochem. J.*, 1939, **33**, 1140.

12 Drill, V. A., unpublished work.

* University Scholar in Biology.

1 Snow, M. and R., *New Phytologist*, 1937, **36**, 1.

auxin applied to the growing regions of certain plants caused union of leaf primordia, and Bausor[2] describes the development of root primordia on stems, petioles, and apical meristem when these parts are treated with beta-naphthoxyacetic acid.

In the present study, seedlings of sunflower *(Helianthus annuus)*, zinnia *(Zinnia elegans)*, and tomato *(Lycopersicum esculentum)* were given moderate X-ray doses (2300 to 2500 r-units for sunflower and zinnia and up to 3000 r-units for tomato). Plants were stunted, and showed rough, warty, and abnormally-shaped leaves, with fasciation of leaves and of stems and frequent fusion of leaf primordia, as previously reported by Johnson.[3] Often, a few weeks after treatment, the meristematic region was divided into 2 or 3 parts, each with a separate group of primordial leaves, showing that the stem was about to branch. Indole-3-acetic acid (0.5 to 1.0%) applied in lanolin paste to seedlings of the above-named species caused increased growth, and with higher concentrations (2%), a fusion of leaves. The meristematic area either resembles that of untreated controls, or various leaf primordia may become joined, or else displaced from normal position. Colchicine (0.5%) applied also in lanolin paste, was found to retard growth, and it induced development of rough, warty, and misshapen leaves just as X-radiation does, while short,

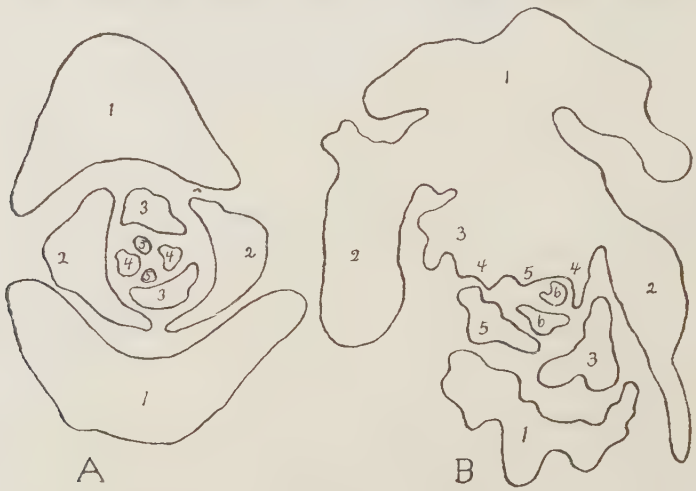

FIG. 1.
Cross sections through primordial regions of sunflower stem. A, control; B, 3 weeks after treatment with beta-naphthoxyacetic acid. Note the distorted and fused primordia. (Numbers on the diagrams indicate leaf pairs.)

[2] Bausor, S. C., *Am. J. Botany*, 1939, **26**, 733.

[3] Johnson, E. L., *Plant Physiology*, 1936, **11**, 319.

TABLE I.
Effects of 2% beta-naphthoxyacetic acid upon sunflower, zinnia, and tomato. (Results given as percentage of plants affected.)

Effects noted	Sunflower	Zinnia	Tomato
Stunted growth	100	100	97
Branching	45	53	25
Distorted leaves	100	100	100
Disturbed phyllotaxy	84	88	86
Fusion of leaves or of leaf segments	89	90	88
Fusion of primordia	75	89	83
Displaced primordia	70	74	70
Splitting of apex	25	33	30
Fasciation	30	34	30

malformed leaf primordia are also produced. The effects of alpha-naphthoxyacetic acid and of beta-naphthoxyacetic acid are similar to the effects of colchicine, but usually less pronounced. The beta form is more potent than the alpha, and since little has been published concerning its action on plants a figure and table are here introduced to describe it.

In general, beta-naphthoxyacetic acid and the other chemicals used in this study are found to produce results upon primordial plant tissues similar to the effects of X-radiation.

11230 P

Droplet Infection of Air: High-speed Photography of Droplet Production by Sneezing.*

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The question of infection of air by droplets given off in coughing and sneezing has received considerable attention during the last few years. In particular, investigation has been directed towards determining the rôle of the air-borne droplet nuclei which result from the evaporation of droplets proper. The bacteriological and epidemiological aspects have recently been discussed by Wells, Wells, and Mudd.[1] Experimentally, little is known of certain of the characteristics of such droplets—their number, size, velocity, settling rate, and rate of

* Contribution No. 163 from the Department of Biology and Public Health.

1 Wells, W. F., Wells, W. W., and Mudd, S., *Am. J. Pub. Health*, 1939, **29**, 863.

evaporation—although these factors are concerned in their dissemination and the production of droplet nuclei. Wells[2] has calculated theoretical settling times and distances of fall before evaporation, for plain water droplets of various sizes suspended in still air of different humidities. These figures are only roughly applicable to actual conditions of air infection, since mouth spray and naso-pharynx droplets will contain some dissolved solids or be mucus-like, and the air will not be still. Rooks[3] has shown experimentally that droplet size is an important factor in nasal filtration, although he knew only the theoretical *differences* in average size of the droplets with which he worked. Weyrauch and Rzymkowski[4] have photographed the *tracks* of moving droplets given off in sneezing and talking. The paths shown indicate that most of the actual droplets travel only relatively short horizontal distances.

We have been able, by means of high-speed photography, to "stop" the motion of droplets given off in coughing and sneezing, thereby permitting measurements of droplet size, velocity, etc. The technic utilizes the light source and control instruments developed by Edgerton, *et al.*,[5] for stroboscopic illumination and high-speed photography. The light source, which is placed at one side of the subject's face, consists of a 9-inch specular reflector with a spiral argon-filled tube through which a 56 microfarad condenser (charged to 2500 volts) discharges. An intense flash of short duration is produced, illuminating the droplets with a dark-field effect so that they stand out sharply even in daylight. The photographically effective duration of flash (exposure time) may be adjusted to the velocity of the particles whose motion is to be stopped. An electrical contact on the camera shutter synchronizes the flash with the shutter motion.

Figure 1 shows the result of a sneeze, at the end of the "downstroke" of the head. Most of the droplets have already been expelled; of these, some 4600 may be counted which were in the focal plane of the camera. This photograph was taken with an ordinary camera on 9 x 12 cm film, with an *f*11 aperture, and an exposure of 1/15,000 of a second. In spite of this short exposure, below the nose may be seen the paths of droplets which moved during that time. (Electrical characteristics of the light source account for the "reversed head and tail" appearance of these particle paths.) Calcula-

2 Wells, W. F., *Am. J. Hyg.*, 1934, **20**, 611.

3 Rooks, R., *Am. J. Hyg.*, 1939, **30**, 7.

4 Weyrauch, F., and Rzymkowski, J., *Z. f. Hyg. u. Infektionskr.*, 1938, **120**, 444.

5 Edgerton, H. E., Germeshausen, K. J., and Grier, H. E., *J. Appl. Physics*, 1937, **8**, 2.

FIG. 1.

Droplets resulting from a sneeze. Instantaneous photograph (exposure 1/15,000 of a second) taken at the end of the "down-stroke" of the head. Note tracks of moving particles beneath the nose, and the string of saliva issuing from the mouth.

tion shows the fastest of these droplets to be moving at a rate of over 100 feet per second. Such a velocity, in dry air, would result in nearly instantaneous evaporation, producing droplet nuclei. The significance of the velocity of expulsion in relation to evaporation is perhaps greater than has been appreciated; it would appear to be a more important factor than settling velocity.

As regards droplet size, optical considerations indicate that only those in sharp focus give photographic images approximating the *true* particle size. The range of *apparent* diameter of the great majority of sneeze droplets, before appreciable evaporation occurs, has been determined from photographic enlargements to be 1/10 to 2 mm, which figures are probably maximum rather than minimum diameters.

We have observed that the involuntary closing of the mouth near the end of a sneeze tends to produce more and smaller droplets, which probably come largely from the saliva in the front of the mouth. Also, the number of droplets issuing from the nose is usually insignificant compared with the number expelled from the mouth. These observa-

tions may be important in relation to infectivity, because of the differences in the microbic flora of the two regions.

Problems of rates of and distances to evaporation, and of minimum droplet size are being investigated.

11231 P

Effect of Repeated Injections of Cobra Venom on Blood Chemistry and Morphology.

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Large doses of cobra venom were repeatedly injected in rabbits for periods varying from 2 to 21 weeks, and quantitative studies were made on the morphology and chemistry of their blood. The venom solution was usually injected 5 times a week and the dosage administered on injection varied from 5 to 10 mouse units. Inasmuch as 5 mouse units constitute the usual therapeutic dose of cobra venom for human beings and 10 mouse units are employed only in more refractory cases, the dosages given rabbits in the present study were manifestly enormous. The results obtained from 10 rabbits are reported in this investigation. Three of the rabbits were used as controls and received no cobra venom while the other 7 received the drug partly by the intravenous and partly by the intramuscular route. Rabbits of different weight were purposely selected at the beginning of the research but all were kept under the same conditions and fed with Purina Chow supplemented with fresh green vegetables. The cobra venom solution (H. W. & D.) employed was prepared in these laboratories and assayed by the authors on white mice by methods described elsewhere.[1] This solution is the same as that employed by various authors in clinical studies published in different journals.[2–6] Crude cobra venom consists largely of neurotoxin and contains relatively small quantities of cytotoxic and proteolytic constituents in striking

[1] Macht, *Proc. Nat. Acad. Sc.*, 1936, **22**, 61.

[2] Macht, *Med. Rec.*, 1936, **144**, 537.

[3] Macht, *Ann. Int. Med.*, 1938, **11**, 1824.

[4] Gayle and Williams, *Southern M. J.*, 1938, **31**, 188.

[5] Rutherford, *New England J. Med.*, 1939, **221**, 408.

[6] Black, *Southern M. J.*, 1940,

contrast to the crude venom of the *Viperidae (Crotalus, Bothrops, Agkistrodon,* and Russell's viper), which are rich in cytotoxic and blood-affecting elements but contain very small amounts of the neurotoxic substance.[7] In the preparation of the cobra venom solution employed by the authors and in the process of sterilizing it, the proteins of the crude substance are almost entirely removed and the cytotoxic and hemolytic constituents are eliminated by at least 50%. The resultant solution of cobra venom therefore consists largely of the neurotoxin which is quite stable and is responsible for the analgesic and other therapeutic properties of cobra venom. This neurotoxin, according to the latest chemical work on the subject, is not a protein but resembles the glucosides in its structure.[8]

In the present study the authors have determined the absolute number of erythrocytes and leukocytes, the differential leukocyte count and the percentage of hemoglobin in the blood. Quantitative biochemical determinations were also made on blood urea, blood urea nitrogen and blood sugar by the usual standard clinical methods (Folin). The results obtained are exhibited in Table I, which indicates the average weight of the animals, the total amount of venom injected in each and the period over which the drug was administered, in addition to the data obtained from the morphological and biochemical examination of the blood. Kidney and liver function tests were also made in these rabbits but are not mentioned in the table as they have already been discussed elsewhere in these PROCEEDINGS.[9] It will be seen that the data set forth in the tables reveals that no specific change occurred in the blood of the treated rabbits as compared with the normal controls and with each other. Certainly no characteristic injuries or pathological effects were noted in the blood examinations. The considerable anemia and high eosinophile count obtained in Rabbit No. 1 were due to the parasitic fungous infection of the ears frequently encountered in laboratory rabbits. The total amount of cobra venom injected in the rabbits varied from 45 to 990 mouse units. None of the rabbits treated with cobra venom revealed any difference in duration of coagulation time. The weight of the animals determined at the end of a period of experimentation differed but slightly from that registered at the beginning of the research. The young rabbits, of course, increased in weight as they grew older but the increment in this case was the same as in the normal controls. Further researches along the above-mentioned

7 Noc, *Ann. Inst. Pasteur,* 1904, **18**, 387.

8 Micheel and Bode, *Naturwissenschaften,* 1938, **26**, 298.

9 Macht and Brooks, PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 418.

TABLE I.

Effect of Cobra Venom on Morphology and Chemistry of the Blood of Rabbits.

Rabbit No.	Avg kg wt of rabbit	No. of weeks rabbit inj.	Total No. mouse units cobra venom inj.	Blood urea	Blood urea N	% of blood sugar	Red blood corpuscles	% of hemoglobin	Leukocyte count	Differential Leukocyte Count						
										Small lymphocytes	Large lymphocytes	Large mononuclears	Polymorphonuclears	Eosinophiles	Neutrophilic myelocytes	Basophiles
1	3.65	13	990	53.5	25.0	0.119	4,176,000	58	14,250	27	7	15	15	32	4	—
2	2.68	12	630	49.2	23.0	0.103	5,600,000	75	18,200	55	14	2	23	6	—	—
3	2.30	3	90	49.2	23.0	0.147	5,632,000	81	17,150	59	13	7	16	1	2	2
4	2.24	21	850	40.0	18.7	0.153	5,368,000	85	8,450	50	16	7	24	2	—	1
5	1.00	Control	0	49.2	23.0	0.111	5,056,000	75	9,600	64	6	3	25	1	—	1
6	4.52	2½	60	61.0	28.5	0.107	5,328,000	78	8,800	39	4	21	33	—	—	2
7	2.00	Control	0	38.7	18.1	0.162	5,984,000	95	10,550	68	5	4	18	3	1	1
8	1.85	7	265	45.8	21.4	0.100	5,840,000	96	14,200	53	11	7	22	3	3	1
9	4.00	Control	0	45.8	21.4	0.111	4,680,000	95	11,200	65	6	5	21	3	—	—
10	3.00	2	45	49.2	23.0	0.133	4,144,000	76	9,250	52	8	6	26	4	4	—

lines are in progress. It is interesting to note that the findings set forth in the present paper regarding rabbits injected with large doses of cobra venom over long periods of time agree with the clinical observations of Steinbrocker, McEachern, La Motta and Brooks[10] in their study on human subjects with material supplied by the senior author of the present paper. These findings agree also with the undetailed report recently published concerning the work of French investigators, who found that no change was produced in the blood sugar level of rabbits but that a rise in blood sugar of guinea pigs was effected by injections of a cobra venom preparation, regarding which details are not given.[11]

Summary. Large quantities of cobra venom were injected in a series of rabbits for periods varying from 2 to 21 weeks. Morphological and biochemical studies on the blood revealed no striking pathological change and no specific effect on the blood picture of the animals as compared with normal controls.

11232 P

Toxicity for Dogs of a Bactericidal Substance Derived from a Soil Bacillus.

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(Introduced by Rene J. Dubos.)

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Dubos[1] has described the isolation from a sporulating soil bacillus of an agent which exerts a powerful bactericidal effect *in vitro* upon Gram positive bacteria, and which if injected intraperitoneally affords protection to white mice infected by the same route with pneumococci or hemolytic streptococci. Subsequently Dubos and Cattaneo[2] reported the results of studies with a protein-free preparation of the bactericidal agent which duplicated the earlier results obtained with the crude protein-containing extract. The present report deals with the toxicity for dogs of a more highly purified protein-free preparation of the bactericidal agent injected by the intravenous route. The

10 Steinbrocker, McEachern, La Motta and Brooks, *J. A. M. A.*, 1940, **114**, 318.

11 Foreign Letter from Paris, *J. A. M. A.*, 1940, **114**, 425.

1 Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 1; Dubos, R. J., *Ibid.*, 1939, **70**, 11.

2 Dubos, R. J., and Cattaneo, C., *J. Exp. Med.*, 1939, **70**, 249.

preparation used was active both *in vitro* and *in vivo* (mice) against Gram positive bacteria.

Short-haired dogs weighing from 8.0 to 12.0 kg were chosen. Prior to the course of injections the animals were observed for from 1 to 2 weeks, and only those showing a normal temperature curve, normal urinalysis and normal blood picture were used. Blood counts and urinalyses were performed daily during the course of injections and at intervals thereafter in the animals which survived. Temperature readings were taken at least twice daily and the animals were weighed frequently throughout the period of observation. Pathological study was made of all dogs which received injections of the bactericidal substance; those animals which did not die as a result of the injections were sacrificed at intervals following treatment. The bactericidal substance was dissolved in alcohol and dilutions of the alcoholic solution in 20 cc of a 5% glucose solution in redistilled water were used for intravenous injection. The doses ranged from 0.05 to 2.0 mg per kg (mg/kg) of body weight per day and injections were continued for 10 days in the dogs which survived. (Fig. 1.)

Six of the 8 animals receiving 0.4 mg/kg or more per day died before the full course of injections was completed, the total dosage varying betwen 1.5 mg and 5 mg/kg. One dog receiving 0.5 mg/kg died on the 42nd day after the course of injections was begun, and one animal which survived the 10 daily injections of 0.4 mg/kg was sacrificed on the 33rd day. None of the 5 dogs died which received a dosage of 0.3 mg/kg or less for 10 days. These were sacrificed

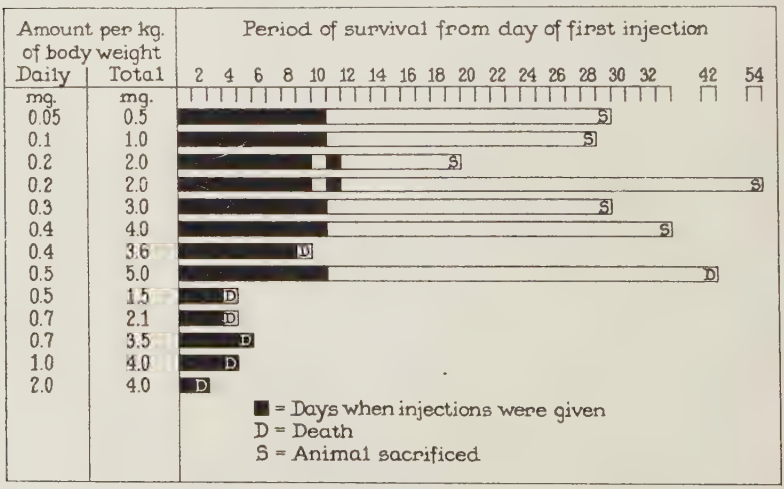

FIG. 1.
Period of survival of dogs receiving varying amounts of the bactericidal substance.

from 23 to 54 days after the course of injections was begun. All of the animals receiving 0.3 mg/kg or more per day showed well-marked toxic manifestations of either acute or chronic nature. In the animals receiving 0.2 mg/kg or less the evidence of toxicity was of minor degree.

The more prominent signs of toxicity were loss of weight associated with anorexia, fever which occurred particularly following the first few injections of the agent, progressive anemia, ascites, hematuria and the excretion of bile in the urine. In the animals which died acutely following injection of the larger doses (0.5 to 2.0 mg/kg) there was marked congestion present in the lungs and abdominal viscera with petechial hemorrhages in the heart, lungs and kidneys. The livers showed acute central necrosis associated with hemorrhage and dilatation of the sinusoids. Diffuse hemorrhage occurred in the spleen, with pronounced phagocytosis of red blood cells by the macrophages. In the kidneys hemorrhage was most marked in the glomeruli. In the animals which received daily 0.3 mg/kg or more and which did not die acutely, the changes in the organs were of a more chronic nature. The liver cells showed fatty degeneration which was most marked about the central veins. In these areas there was an increase in the reticular tissue, but cirrhotic changes were minimal. In 2 of the animals ascites was present. The only change noted in the organs of the animals receiving 0.2 mg/kg or less was a slight degree of fatty degeneration of the liver.

Summary. A study has been made of the toxicity for dogs of a protein-free preparation of the bactericidal substance described by Dubos when injected by the intravenous route. Seven of the 8 animals which received 0.4 mg/kg or more daily died as a result of the injections, and in 6 of these death occurred before the course of 10 daily injections was completed. All animals receiving 0.3 mg/kg or more showed well-marked acute or chronic changes in the liver, spleen, kidneys, heart and lungs. Animals which received daily 0.2 mg/kg or less for 10 days showed only minor evidence of toxicity.

11233

Failure of Hypnotic and Convulsive Agents to Alter the Course of Experimental Poliomyelitis.

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Little is known concerning the metabolism of the central nervous system in its relation to the factors which support or inhibit the propagation of neurotropic viruses. Yet it would seem that better knowledge of the chemical reactions which underlie the interaction between virus and substrate should materially contribute to our understanding of the infectious process itself. With the present analytical methods it is doubtful that a direct biochemical approach would yield any more concise data than are already on hand regarding such changes as disturbance in the operation of the oxidation-reduction potential of virus-infected nerve tissue.[1, 2] However, an indirect attack of the problem may be feasible by studying the effect of comparatively crude alterations in the central nervous system—brought about by physiological or pharmacological methods—on the evolution of the characteristic virus lesion.

One recent attempt in this direction was made by Howe and Bodian.[3] By section of the axone, these authors evidently succeeded in inducing an irreversible change in the metabolism of the nerve cell which rendered the affected territory relatively refractory to subsequent invasion by poliomyelitis virus. Certain drugs also cause a profound derangement in the metabolic function of the central nervous system, particularly the narcotics and hypnotics, which are capable of inhibiting the oxidation of substances essential in carbohydrate metabolism.[4-7] The possible usefulness of drugs of this type in modifying the action of neurotropic agents of disease has not yet been explored, except for the observation that death from botulism can be delayed in guinea pigs when these animals are subjected to

[1] Brodie, M., and Wortis, S. G., *Arch. Neurol. and Psych.*, 1934, **32**, 1159.

[2] Jungeblut, C. W., and Feiner, R. R., *J. Exp. Med.*, 1937, **66**, 479.

[3] Howe, H. A., and Bodian, D., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 346.

[4] Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, 1931, **25**, 117; 1934, **28**, 1521.

[5] Emerson, G. A., Proc. Soc. Exp. Biol. and Med., 1935, **33**, 171.

[6] Dameshek, W., Myerson, A., and Loman, J., *Am. J. Psych.*, 1934, **91**, 113.

[7] Wortis, S. B., *Am. J. Psych.*, 1936, **93**, 87.

ether anesthesia or placed under the influence of sedatives.[8] More recently the property of excessive doses of insulin and metrazol to elicit shock or convulsions has been put to effective therapeutic use in the treatment of certain nervous disorders. The precise mechanism of this form of therapy is still obscure. Gross pathological changes in the brain, which occur as the result of shock, indicate severe structural damage to nerve cells;[9-15] there is also evidence of depressed cerebral metabolism since hypoglycemic shock diminishes the oxygen utilization of brain tissue through the absence of available dextrose,[16, 17, 18] whereas metrazol convulsions achieve the same effect by decreasing the oxygen necessary for the combustion of this sugar.[19, 20, 21] The resulting anoxemia may act by stimulating the sympathetic system,[22] but it may also affect the brain directly by increasing cellular permeability.[23] In view of what has been said above, it was decided to determine whether the protracted administration of a powerful hypnotic, *i. e.*, luminal, or the production of systemic shock, by means of insulin or metrazol, had any effect on the course of poliomyelitis in the monkey.

Experiment I. Effect of luminal depression: One Rhesus monkey, weighing about 2000 g, received daily doses of 100 to 150 mg sodium luminal (sodium salt of phenyl-ethyl-malonyl urea) by the subcutaneous route, beginning 3 days before intracerebral infection with poliomyelitis virus and continued for 7 days after infection. An-

8 Bronfenbrenner, J., and Weiss, H., *J. Exp. Med.*, 1924, **39**, 517.

9 Schmid, H., *Ann. Méd.-psychol.*, 1936, **94**, 658.

10 Weil, A., Liebert, E., and Heilbrunn, G., *Arch. Neurol. and Psych.*, 1938, **39**, 467.

11 Baker, A. B., *Arch. Pathol.*, 1938, **26**, 765.

12 Weil, A., and Liebert, E., *Arch. Neurol. and Psych.*, 1938, **39**, 1108.

13 Baker, A. B., *Am. J. Psych.*, 1939, **96**, 109.

14 Yannet, H., *Arch. Neurol. and Psych.*, 1939, **42**, 395.

15 Ferraro, A., and Jervis, G. A., *Am. J. Psych.*, 1939, **96**, 103.

16 Holmes, E. G., *Biochem. J.*, 1930, **24**, 914; 1932, **26**, 2010.

17 Gellhorn, E., *J. Am. Med. Assn.*, 1938, **110**, 1433.

18 Wortis, S. B., *N. Y. State J. Med.*, 1938, **38**, 1015.

19 Himwich, H. E., Bowman, N. M., Fazekas, J. F., and Orenstein, L. L., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 359.

20 Low, A. A., Sonenthal, I. R., Blaurock, M. F., Kaplan, M., and Sherman, I., *Arch. Neurol. and Psych.*, 1938, **39**, 717.

21 Himwich, H. E., Bowman, N. M., Wortis, J., and Fazekas, J. F., *J. Am. Med. Assn.*, 1939, **112**, 1572.

22 Gellhorn, E., *Arch. Neurol. and Psych.*, 1938, **40**, 125.

23 Spiegel, E., and Spiegel-Adolf, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 834.

other monkey of approximately the same weight was subjected to similar treatment, except that the drug was administered first on the day of infection and maintained thereafter. The amount of luminal given to both animals sufficed to induce profound stupor which lasted for several hours. One control monkey, infected with the same dose of virus (0.01 cc of a 5% suspension of the RMV strain) but left without drug treatment, accompanied this experiment. The results are given in Table I. It will be seen that both experimental animals developed complete paralysis in approximately the same length of time as did the control animal. Moreover, the cord lesions were identical in all 3 monkeys.

Experiment II. Effect of hypoglycemic shock: A total of 13 animals were used in this experiment and arranged in 3 groups. The first group consisted of 3 monkeys which received daily doses of from 3 to 8 units of insulin by the intravenous route, beginning 3 days before intracerebral infection with poliomyelitis virus and discontinued after infection. The second group was made up of a total of 6 monkeys, all of which were injected daily with similar doses of insulin, beginning 3 days before infection and maintained thereafter for 6 days. The third group contained 4 monkeys in which insulin treatment of similar dosage was begun on the day of infection and continued daily for a period of 4 days. The above experiment included 3 control monkeys, infected with the same dose of virus (0.01 cc of a 5% suspension) but left without treatment. The amount of insulin used in these tests was sufficient to lower the blood sugar to the convulsive level of about 30 mg %. However the degree of systemic response varied widely among different monkeys, doses of insulin which, upon first administration, induced severe shock in some animals, failing to produce the same effect in others except as the result of cumulative injections. In some instances of extremely violent symptoms it became necessary to resort to glucose administration in order to prevent coma and death. Because of these irregularities the number of shocks and their intensity varied considerably throughout the whole series but the dosage was individualized to the extent that each monkey experienced at least 3 moderately severe

TABLE I.
Effect of Narcotic Doses of Luminal on Experimental Poliomyelitis.

Monkey No.	Dose of Luminal Sc., mg	Type of treatment	Dose of virus (5% suspension) Ic.	Result
1	100-150	before and after infection	0.01 cc	Complete paralysis, 9 days
2	100-150	after infection	,,	,, ,, 7 ,,
3	—	—	,,	,, ,, 8 ,,

hypoglycemic shocks during the entire course of the experiment. The results are listed in Table II. It appears from this table that the progress of the disease in the insulin-treated monkeys deviated in no significant way from that observed in the control animals. Likewise, histological examination revealed no fundamental difference in the distribution or severity of the cord lesions between the treated and untreated group.

Experiment III. Effect of metrazol shock: One Rhesus monkey of 2000 g weight was injected intravenously with daily doses of 0.3 to 0.6 cc metrazol (1 cc = 100 mg of penta-methylene-tetrazol in 1% sodium phosphate solution) for a period of 4 days following intracerebral infection with poliomyelitis virus. Another monkey of similar weight received daily injections of the same dosage of the drug, beginning 5 days before poliomyelitic infection and discontinued thereafter. Two control monkeys, infected with corresponding doses of virus (1 cc or 0.1 cc of a 5% virus suspension) but left untreated, completed this experiment. The dose of metrazol was large enough to induce in both animals violent clonic-tonic convulsions which set in a few seconds after injection of the drug and lasted for from 5 to 10 minutes. The results are given in Table III. It will be noted that paralysis developed in the treated and untreated

TABLE II.
Effect of Insulin Shock on Experimental Poliomyelitis.

Monkey* No.	Dose of insulin Iv.	Type of treatment	Complete paralysis in
4	3-8 units	before infection	8 days
5	"	" "	8 "
6	"	" "	12 "
7	"	" and after infection	6 "
8	"	" " " "	7 "
9	"	" " " "	7 "
10	"	" " " "	8 "
11	"	" " " "	9 "
12	"	" " " "	9 "
13	"	after infection	6 "
14	"	" "	6 "
15	"	" "	6 "
16	"	" "	9 "
17	—	—	8 "
18	—	—	7 "
19	—	—	7 "

*All monkeys were infected intracerebrally with 0.01 cc of a 5% suspension of virus.

TABLE III.
Effect of Metrazol Shock on Experimental Poliomyelitis. Complete paralysis in 5 days.

Monkey No.	Dose of metrazol Iv.	Type of treatment	Dose of virus (5% suspension) Ic.
20	0.3-0.6 cc	before infection	0.1 cc
21	"	after infection	1 "
22	—	—	0.1 "
23	—	—	1 "

group with no perceptible difference. Upon autopsy all monkeys showed severe lesions in the cord.

Conclusions. Neither the administration of narcotic doses of luminal nor the production of systemic shock by means of insulin or metrazol were capable of influencing the course of experimental poliomyelitis. Moreover, the extent and severity of the lesions in the spinal cord showed no significant difference between treated monkeys and untreated control animals. Even though essentially negative, the above results are considered important in demonstrating that propagation of the virus of poliomyelitis in the central nervous system is not affected by profound cytological and metabolic changes in the nerve tissue, as were produced by the methods employed in this work.

11234

On the Detoxication of Phenylacetic Acid by Glucuronic Acid in Humans.

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Using reduction methods, Quick[1] working with dogs, concluded that phenylacetic acid is excreted in the form of its glucuronide to the extent of 34%; and a considerable portion of the remainder appeared in conjugation with glycine. Working with human subjects, and using similar methods of determination, Ambrose, Power, and Sherwin[2] claimed that by far the largest quantity eliminated is in combination with glutamine, whereas only 5% appears to be conjugated with glucuronic acid.

1 Quick, A. J., *J. Biol. Chem.*, 1928, **77**, 581.

2 Ambrose, A. A., Power, F. W., and Sherwin, C. P., *J. Biol. Chem.*, 1933, **101**, 669.

In estimating the quantity of glucuronides in the urine, Quick, Sherwin, etc., used reduction methods[3] and assumed that the increase in reducing substances in the urine, produced as a result of the ingestion of phenylacetic acid, was due solely to an increase in urinary glucuronides.

Realizing the inadequacy of such reduction methods, Sherwin[4] wrote: "Unfortunately, many of the studies in glucuronic acid have been confined to a qualitative or 'quantitative' test for reducing substances in the urine, and attempts to check many of these results have not proved satisfactory." For this reason, and because of the importance of glucuronic acid in processes dealing with detoxication—the ingestion of phenylacetic acid has been suggested as a liver functional test—we were interested in studying the detoxication of phenylacetic acid by glucuronic acid, using a *direct* method for the determination of glucuronides. Fortunately, this was made possible by the work of Maughan, Evelyn, and Browne,[5] who devised a photo-

TABLE I.
Amounts of Glucuronic Acid Eliminated.

	Mg glucuronic acid per 24-hr sample Days								
Subject	1	2	3	4	5*	6	7	8	9
A	496	534	341	437	965	539	414	440	423
B	604	568	627	643	1115	382	476	651	465
C	418	401	530	496	992	617	520	425	421
D	502	551	615	537	1078	369	333	378	448
E	289	471	398	377	951	405	442	486	535
F	528	545	499	480	1154	477	549	549	583
G	470	503	565	658	1067	664	369	516	423
H	623	554	708	717	996	588	574	602	

*Ingestion of 5 g of phenylacetic acid at the beginning of the 5th day.

TABLE II.
Increase in Glucuronic Acid in the Urine as a Result of Feeding Phenylacetic Acid.

Subject	Avg mg glucuronic acid per 24 hr in normal urines	Increase in mg glucuronic acid on ingestion of 5 g of phenylacetic acid
A	453	512
B	540	575
C	479	513
D	467	611
E	425	526
F	526	628
G	521	546
H	624	372

3 Somogyi, M., *J. Biol. Chem.*, 1926, **70**, 599.

4 Harrow, B., and Sherwin, C. P., *A Text-Book of Biochemistry*, 1935, p. 380.

5 Maughan, G. B., Evelyn, K. A., and Browne, J. S. L., *J. Biol. Chem.*, 1938, **126**, 567.

electric method for the quantitative estimation of glucuronic acid and conjugated glucuronides in human urine. They made use of the color developed by the reaction of the glucuronide with naphthoresorcinol on being heated with hydrochloric acid.

The experimental procedure was as follows: 24-hour urine samples of normal male subjects were collected for 4 days. On the fifth day, 5 g of phenylacetic acid—which is as much as can be conveniently tolerated—previously neutralized with 0.2 molar NaOH, were ingested. The urine was collected for the next 5 days. The amount of glucuronide was determined in each 24-hour sample according to the method previously referred to.[5] The analyses were carried out in a photoelectric colorimeter of the type described by Withrow, Shrewsbury, and Kraybill.[6] The results with 8 subjects are shown in Tables I and II.

Conclusion. Dealing first with normal (control) urines, the amount of glucuronic acid in 24-hour samples varied from 350-650 mg, with an occasional lower or higher figure. These results agree well with those obtained by Maughan, Evelyn, and Browne[5] and by Roe and Hall.[7] Next, with regard to the effect of ingesting 5 g of phenylacetic acid, we find that this results in an average increase of 535 mg, an amount which corresponds to a detoxication by glucuronic acid of 7.5% of the phenylacetic acid ingested.

11235 P

Metabolic Studies in Dermatomyositis, with a Note on the Effect of Wheat Germ.*

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Patients with dermatomyositis frequently have extensive changes in the skin, muscles and bones. In addition, many patients have symptoms of Raynaud's Disease. The muscular disability in dermatomyositis can be as marked as that occurring in advanced pro-

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* Aided in part by a grant from the National Foundation for Infantile Paralysis, Inc.

gressive muscular dystrophy with extreme wasting and weakness. On radiographic examination, the bones often show areas of rarefaction, and in some instances complete absorption of the distal phalanges of the fingers. Deposits of calcium in the subcutaneous tissues are a frequent finding. Despite the extensive lesions that occur in dermatomyositis, few studies of the metabolism of patients with this condition have been made.

The object of the present investigations was to obtain a clearer understanding of the changes occurring in the muscles and bones. The studies included complete balances of calcium, phosphorus and magnesium at different levels of intake. In addition, the urinary excretion of creatinine and creatine was determined daily over periods of several months while the patients were receiving various substances that were being studied for their possible therapeutic effects.

Observations and Discussion. Calcium, Phosphorus and Magnesium: The data on the balance of calcium and phosphorus are shown in Table I. The most significant finding was the relatively low urinary excretion of calcium in both patients. Whereas the ratio of urinary calcium to fecal calcium in normal subjects usually is of the order of 1:5, the ratio in both patients was about 1:13. The metabolism of phosphorus is of interest, although probably of less significance, in that there appeared to be a diminished ability to store phosphorus when the daily intake was around 2.0 g. The magnesium balance was normal.

Creatinine and Creatine: The data on the metabolism of creatinine and creatine are presented briefly in the chart (Fig. 1). In both patients, the urinary excretion of creatinine was diminished, and ap-

TABLE I.

Balance of Calcium and Phosphorus at Different Levels of Intake.

There were 5 days in each period during which the diet was constant. Each period was preceded by a preliminary period of 5 days during which the diet was similar to that of the experimental period.

		Intake		Output				Balance	
				Urine		Feces			
Patient	Period	Ca mg	P mg	Ca mg	P mg	Ca mg	P mg	Ca mg	P mg
1	I	1149	1485	61	830	806	368	+282	+287
	II	102	627	30	467	214	197	—142	— 37
	III	2016	1900	92	1222	1560	640	+364	+ 38
2	I	1016	1441	49	800	760	458	+207	+183
	II	102	627	21	454	188	178	—107	— 3
	III	2007	2060	96	1205	1700	888	+211	— 33

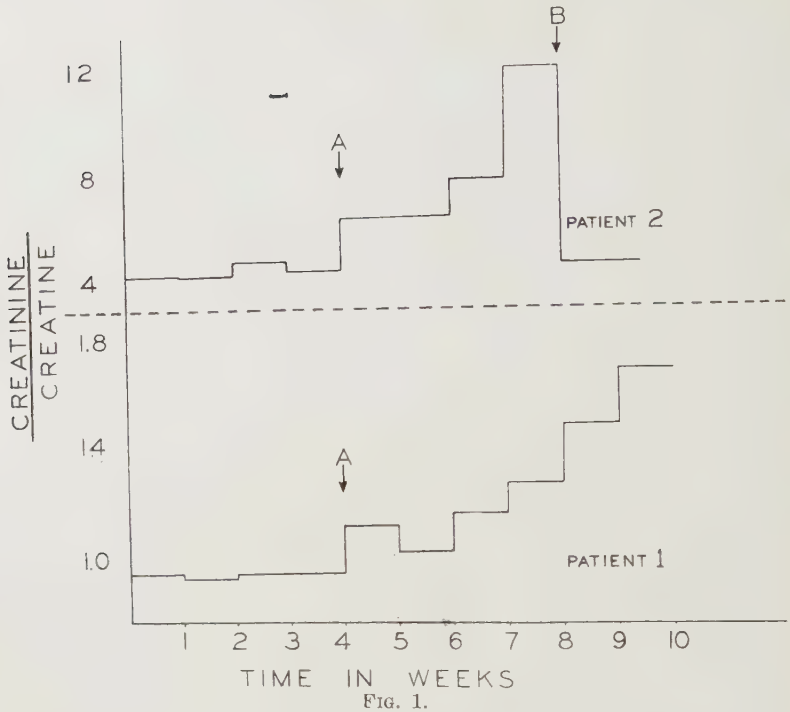

FIG. 1.

Effect of wheat germ on the ratio, $\frac{\text{urinary creatinine}}{\text{urinary creatine}}$. Administration of 125 g wheat germ daily started at "A," and stopped at "B."

peared to be proportional to the amount of muscular wasting. In Patient 1, both the amount of muscular wasting and the decrease in urinary creatinine were of the same order as those seen in patients in the advanced stages of progressive muscular dystrophy. In this patient the amount of daily creatinine was only 0.300 g, and the creatinine index was only 7.

Both patients excreted considerable amounts of creatine and showed an impaired ability to retain ingested creatine. Patient 1, with the more extensive muscular changes, excreted the larger amounts, namely, around 0.350 g daily, and of an ingested dose of creatine only 30% was retained.

Effect of Wheat Germ: Wheat germ was found to have a significant effect on the metabolism of creatine and creatinine in both patients (chart). The patients each were given 125 g of wheat germ mixed in tomato juice daily. After a period of about 2 weeks, there was a striking and progressive decrease in the creatinuria with a

slower rise in the creatinine excretion. Concomitant with these metabolic changes, the clinical condition of the patients improved steadily. Muscular function increased, the tightness of the skin diminished, and the patients were able to perform muscular tasks that had been impossible for a long time.

In one series of experiments a year ago the wheat germ was incubated with normal gastric juice at 37°C for about one hour before it was administered. Patient 1 showed a definite response to wheat germ prepared in this manner. However, untreated wheat germ appeared to be equaly effective when given in large amounts. When the administration of wheat germ to Patient 2 was discontinued, the creatinuria increased to the level observed before the period when wheat germ was given.

The parenteral administration of riboflavin and vitamin B_6 daily for 6 weeks was without effect.

Patients with dermatomyositis often improve spontaneously. However, the results of this study are suggestive and warrant further investigations along these lines. Since dermatomyositis is a comparatively rare condition it is hoped that other workers will study these effects of wheat germ.

Summary. Two patients with dermatomyositis showed a decreased excretion of urinary calcium and an apparent impaired ability to store phosphorus. The amounts of urinary creatinine were diminished proportionally to the amounts of muscular wasting. Both patients had considerable creatinuria and an impaired creatine tolerance. Prolonged administration of large amounts of wheat germ was followed by decrease in creatinuria, increase in urinary creatinine, and definite clinical improvement.

11236 P

Effect of Whole Bile and Bile Salt of Swine on Gastric Motility of the Dog.

JAMES M. WINFIELD. (Introduced by T. L. Patterson.)

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While carrying on a clinical evaluation of the effects of feeding dried whole bile to patients suffering from a variety of lesions it was noted that the symptom of anorexia was often relieved.[1] Sensations of hunger occurred within a period of a few minutes to a few hours although in an occasional instance anorexia has been increased. Because of these observations we conceived the possibility that gastric contractions might be affected, or even called forth from a quiescent stomach. Accordingly, we determined to investigate the response to dried bile of the stomach of fasting dogs.

Method. Three dogs were prepared by operation with gastric fistulae after the method of Carlson[2] and after training and conditioning the animals, the gastric tonus and motility was measured by the balloon-manometer method. A condom balloon 8-10 cm in length was attached to the end of a No. 10 catheter and a No. 8 catheter attached alongside for gastric injection. The catheters and balloon were inserted through the gastrostomy opening into the fundus of the dog's stomach and then 50 cc of air was injected into the balloon and the catheter connected to a bromoform manometer. The 50 cc of air usually created a pressure of 3-6 cm.

The gastric tonus and motility were then recorded by Patterson's kymographic ink recording method.[3]

The dried whole bile used in the experiments was swine gallbladder bile prepared by vacuum distillation at low temperature.* As sodium α-glycohyodesoxycholate is the principal bile salt in swine bile,[4] it was decided to test the effect of this salt as well as whole bile.

From analysis of the results of the foregoing preliminary study it would seem that when either dried whole swine bile or sodium α-gly-

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* Supplied by Parke, Davis and Company.

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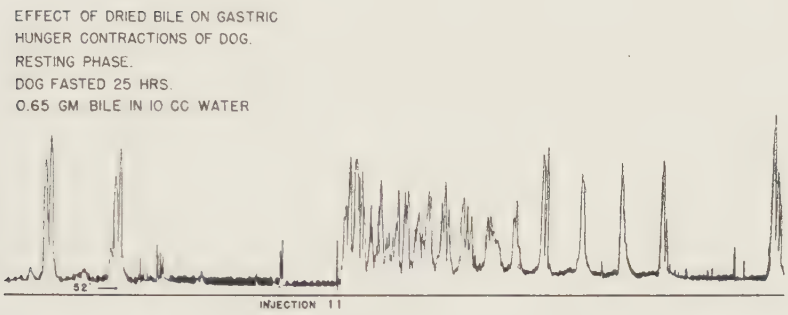

FIG. 1.

Note immediate effect, increase in tone and production of forceful contractions after injecting whole bile into stomach during quiescent phase.

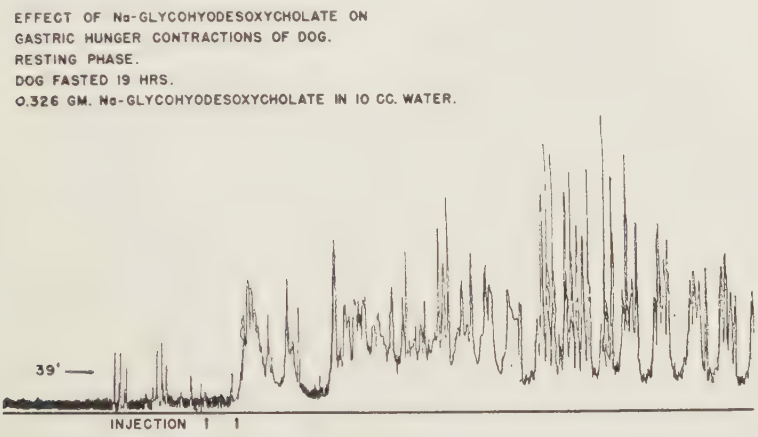

FIG. 2.

Note immediate effect, increase in tone and production of forceful contractions after injecting sodium α-glycohyodesoxycholate into stomach during quiescent phase. Effect the same as obtained with whole bile.

cohyodesoxycholate dissolved in water are placed in a dog's stomach during the quiescent phase gastric hunger contractions are produced (Figs. 1, 2). This response occurred in all of 24 experiments using whole bile and in all of 5 experiments using the bile salt.

On the contrary, when dried whole swine bile was placed in a dog's stomach during the contraction phase, it apparently caused a definite but relatively short inhibition of contractions in 21 of 34 experiments.

The same inhibitory effect on gastric contractions was noted in 6 of 13 experiments when sodium α-glycohyodesoxycholate was used. The effect, however, was less marked than that obtained with whole bile.

Introduction of plain water to the amount of 10-15 cc caused mild inhibition of gastric hunger contractions in 8 of 26 times attempted.

This effect is much the same as that obtained with the bile and sodium α-glycohyodesoxycholate solutions although the bile inhibition occurred somewhat more consistently.

If the stomach happens to be in a quiescent phase, the production of contractions might conceivably explain the prompt hunger sensations and relief of anorexia seen clinically.

The mechanism is not clearly understood as yet. Whether the response occurs because of an irritative phenomenon on the mucosa of the stomach or because of an effect on the intrinsic nerves cannot be stated at present.

11237 P

Attempts to Demonstrate Poliomyelitis Virus in Extraneural Tissues.*

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The recovery of the virus of poliomyelitis from nasopharyngeal washings[1] and the recent positive results with sewage,[2] stools of poliomyelitis patients[1, 3, 4] and healthy carriers[5, 6] has again raised the question of virus distribution in tissues other than those of the CNS. Studies with experimental animals have demonstrated the presence of viruses such as rabies[7] and poliomyelitis[8–12] in the extraneural tissues following intracerebral or intravenous injections. In the present work an attempt was made to recover the virus of polio-

* This work was aided by a grant from the Clara Ward Seabury Clinic for Infantile Paralysis.

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2 Paul, J. R., Trask, J. D., and Gard, S., *J. Bact.*, 1940, **39**, 63.

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TABLE I.
Distribution of Poliomyelitis Virus in Human Tissues.‡

Patient No.	Tissues tested	Neurological signs in monkeys		Pathology in monkeys
		First passage	Second passage	
A-2130	Spleen	Paresis lt. arm and leg—5 days	2 unsuccessful attempts	Small abscess at inoculation site. No microscopic lesion in spinal cord.
	Liver	Negative	*	†
A-2131	Spleen	”	*	†
	Liver	”	*	†
A-2136	Spleen	Flaccid paralysis post. extremity 5 days	1 unsuccessful attempt	Small abscess at inoculation site. No microscopic lesion in spinal cord.
	Liver	Negative	*	†
A-2139	Spleen	Brain abscess	*	†
	Liver	Negative	*	†
	Mesenteric nodes	”	*	†
A-2141	Spleen	”	*	†
	Liver	”	*	†
	Mesenteric nodes	”	*	†
A-2162	Spleen	”	*	†
	Liver	”	*	†
	Mesenteric nodes	”	*	†

* Second passage not attempted.

† No pathological examination made.

‡ We are indebted for autopsy tissues to the Pathology Department of Herman Kiefer Hospital, Detroit, Mich., Joseph A. Kasper, Director.

myelitis from the tissues of a monkey injected intravenously 5 days earlier with the Aycock strain. The animal, which showed no neurological signs, was exsanguinated and perfused with 1 liter of 0.85% saline solution. Lung, liver, spleen, intestine, brain and spinal cord were removed, ground separately, and diluted with saline. Anesthetic ether was added to a final concentration of 20%, and the suspensions were stored overnight at 5°C. The supernatant fluids were decanted, reduced *in vacuo* to one-third their original volume, and 1.5 cc of each extract inoculated intracranially into *Macacus rhesus* monkeys. The liver and spleen extracts were pooled previous to injection. The animals which received this material and the extract of lung developed quadriplegia in 9 and 24 days respectively. The other animals remained normal. The histopathology in both cases was that of acute poliomyelitis. Second transfers were successful.

Before this work could be evaluated, an unusual amount of tissue became available from human autopsies.† This material was subjected to similar treatment and injected intracranially into monkeys (Table I). The monkeys receiving spleen extracts A-2130 and A-2136 developed neurological signs, but the histopathology and monkey transfers were negative. Small pyogenic abscesses were present in both animals at the site of inoculation in the frontal region and may have caused the flaccid paralysis.[13]

Conclusions. The technic described, while similar to that used with fair results for the isolation of the virus from stools and nasopharyngeal washings,[1] failed to detect the agent in extraneural tissues of poliomyelitis patients. Lennette[14] has suggested that the virus may be present outside the CNS, but unidentifiable because of an attachment with antibody. Definite conclusions regarding the presence or absence of virus in extraneural tissues must await the development of more precise methods.

† From the Detroit epidemic, July through October, 1939.

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11238

Susceptibility of the Eastern Cotton Rat (*Sigmodon hispidus littoralis*) to Diphtheric Toxin.

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The high degree of natural resistance of the albino rat *(Mus norvegicus albinus)* to diphtheric infection and intoxication is a matter of common knowledge. While there may be some uncertainty as to the precise amount which rats will tolerate, all investigators agree that many times the lethal dose for guinea pigs of toxin or of virulent bacilli can be injected into rats without causing death.[1] Armstrong[2] reported that a variety of Eastern cotton rats *(Sigmodon hispidus hispidus)* are apparently susceptible to infection with poliomyelitic virus. There are no data on the reaction of the cotton rat to diphtheric infection or intoxication. In view of a number of previous observations, all of which suggest some relationship between susceptibility to diphtheria and to poliomyelitis,[3] our interest was aroused to determine whether these rodents are as refractory to experimental diphtheria as is the albino rat.

The cotton rats were obtained in several shipments from a dealer in Florida who had trapped the wild animals in the field. They appeared to be a variety of Eastern cotton rats, listed as *Sigmodon hispidus littoralis*, which is closely related to *Sigmodon hispidus hispidus*. They were sturdy specimens, weighing from approximately 70 to 120 g, and thrived when placed on a diet consisting of oatmeal, peanuts, green vegetables and apples. However, upon necropsying animals that had died as the result of experimentation, it was found that they were, as a rule, heavily infected with intestinal and pulmonary worms and occasionally carried trypanosomes in their blood. The latter observation will make the subject of another

1 Diphtheria Monograph, Medical Research Council, London, 1923.

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3 (a) Zingher, A., *Am. J. Dis. Childr.*, 1917, **13**, 247; (b) Jungeblut, C. W., *J. Immunol.*, 1934, **27**, 17; (c) Foley, A. R., *Canad. Publ. Health J.*, 1934, **25**, 260; (d) Jungeblut, C. W., *Am. J. Med. Sci.*, 1936, **192**, 661; (e) Fischer, A. E., and Stillerman, M., *Am. J. Dis. Childr.*, 1937, **54**, 984; (f) Dwyer, J. M., *Med. J. Australia*, 1938, January 8, p. 52; (g) Heaslip, W. G., *Austral. J. Exp. Biol. and Med. Sci.*, 1938, **16**, 285; (h) Burnet, F. M., Freeman, M., Jackson, A. V., and Lush, D., *Med. J. Australia*, 1939, August 5, p. 198.

TABLE I.
Infection with *C. diphtheriæ in the Guinea Pig, Cotton Rat, and Albino Rat.*
(Subcutaneous injection of bacillary suspension.)

C. diphtheriæ, gravis strain		Guinea pigs No.	Result		Cotton rats No.	Result		Albino rats No.	Result	
			Died	Lived		Died	Lived		Died	Lived
1	slant	1	1 (24h)	0	1	1 (48h)	0	1	0	1
1/2	''	1	1 ''	0	1	1 (4d)	0	1	0	1
1/5	''	1	1 ''	0	1	1 ''	0	1	0	1
1/10	''	1	1 (48h)	0	2	2 (4d, 6d)	0	2	0	2
1/20	''	1	1 ''	0	1	1 (5d)	0			
1/40	''	1	1 ''	0	1	0	1			
1/100	''	1	0	1	1	0	1			
1/200	''	1	0	1						

detailed report by Dr. Culbertson from this laboratory. We are mentioning these facts briefly at this time since they may possibly have a bearing on the resistance of this animal to experimentally induced infections and intoxications. So far no laboratory-bred cotton rats, free from intercurrent disease, have been available.

Diphtheric infection: A strain of *C. diphtheriæ* of known high virulence was used for this work (London *gravis* I). We are indebted to Dr. Martin Frobisher, Jr., of Johns Hopkins University for kindly sending us this strain. Cultures grown for 24 hours on Lœffler slants in 4″ x ½″ tubes were suspended in saline and the bacterial emulsion, in graded doses, was injected subcutaneously into guinea pigs, cotton rats, and albino rats. The results are given in Table I. It appears from these tests that amounts as small as 1/40 of a slant killed guinea pigs uniformly within 48 hours, whereas 1/20 of a slant sufficed to cause death in the cotton rat within 5 days. No deaths occurred in a series of albino rats injected with amounts varying from 1/10 of a slant to as much as a full slant. The lesions in guinea pigs, both local and in the adrenal, were pathognomonic of diphtheric death. A similar characteristic local lesion consisting of a gelatinous, hemorrhagic exudate was but rarely obtained in the cotton rat; more often there was evidence of a localized abscess at the point of in-

FIG. 1.

Local lesion in the cotton rat following subcutaneous injection of culture of *C. diphtheriæ*.

jection which yielded a pure culture of diphtheria bacilli upon transfer to suitable media. This lesion is well illustrated in photograph 1. Adrenal involvement occurred irregularly and usually was not very intense. Such abscesses as have just been described were regularly seen in surviving albino rats when these animals were sacrificed during the early intervals following infection. Systematic observations on the type of cellular response of the albino rat to diphtheric infection and subsequent changes in the morphology and virulence of the bacilli will be reported elsewhere.

Diphtheric intoxication: The toxin employed was a sample of Park-Williams No. 8 diphtheric toxin and was obtained through the courtesy of the New York City Department of Health. At the time of running these tests, the minimal fatal dose for guinea pigs was 1 cc of a 1:700 dilution. Doses of toxin ranging from 2000 mfd to 1 mfd were injected subcutaneously into guinea pigs, cotton rats, and albino rats. The results are brought together in Table II. It will be seen from the figures that cotton rats succumbed uniformly to amounts of toxin as small as 5 mfd; further reduction to 2 mfd caused the death of 2 out of 4 cotton rats, while a similar number of animals survived a single fatal dose for the guinea pig. As might have been expected, albino rats tolerated inordinately larger amounts of toxin, succumbing only to 1000 and 2000 mfd. In harmony with the results obtained with bacillary infection, the fully developed picture of the local lesion, which is so characteristic for guinea pigs, was rarely seen in the cotton rat; moreover, adrenal congestion usually was not very marked and was occasionally absent. Despite prolonged observation, surviving cotton rats never exhibited any signs of postdiphtheric paralysis, excepting one animal which showed flaccid paralysis of both hind legs 6 weeks following injection with 1 mfd of toxin; this animal died 2 days later.

The above results clearly indicate the high systemic susceptibility of the cotton rat to diphtheric intoxication. The objection might be raised that some of our animals were abnormally sensitive to the toxin because a certain percentage were simultaneous carriers of intestinal parasites and of trypanosomes. Such an assumption, however, would hardly explain the closely graded correspondence, throughout the entire experimental series, between the size of the inoculum—be it toxin or culture—and the response of the animal, as measured by the length of its survival. In order to throw more light on this problem another experiment was carried out in which we studied the local effect of the toxin following endermal injection in

TABLE II.

Effect of Diphtheric Toxin Injected Subcutaneously into Guinea Pigs, Cotton Rats, and Albino Rats.

Diphtheria Toxin, mfd	Guinea pigs, No. of animals	Result		Cotton Rats No.	Result		Albino Rats No.	Result	
		Died	Lived		Died	Lived		Died	Lived
2000							2	2 (5d, 6d)	0
1000							2	2 " "	0
700				1	1 (24h)	0	2	0	2
350				1	1 "	0	2	0	2
200				1	1 (36h)	0	2	0	2
100				2	2 (36h, 36h)	0			
50				2	2 (60h, 72h)	0			
25				3	3 (72h, 72h, 72h)	0			
10				3	3 (72h, 5d, 5d)	0			
5				4	4 (5d, 8d, 8d, 10d)	0			
2				4	2 (8d, 8d)	2			
1*	4	4 (48h, 48h, 72h, 96h)	0	4	0	4†			

*Represents 1 cc of a 1:700 dilution of toxin.

†See text for development of post-diphtheric paralysis and late death in one animal.

TABLE III.

Effect of Diphtheric Toxin Injected Endermally into Guinea Pigs, Cotton Rats, and Albino Rats.

Animal	Interval	Mfd of toxin injected endermally, each dose being contained in a uniform volume of 0.1 cc						
		1	1/2	1/5	1/10	1/50	1/100	1/200
Guinea Pig No. 1	24h				+	+	+	+
	72h				++++	++++	++	++
	5d				++++	++++	++	++
" " No. 2	24h				+	+	+	+
	72h				++++	++++	+++	++
	5d				++++	++++	+++	+
Cotton Rat No. 1	24h				0	0	0	0
	72h				0	0	0	0
	5d				0	0	0	0
" " No. 2	24h	0	0	0	0			
	72h	+	+	±	±			
	5d	++++	++++	±	±			
" " No. 3	24h	0	0	0	0	0		
	72h	±	0	0	0	0		
	5d	+++	±	±	±	0		
Albino Rat No. 1	24h	0	0	0	0	0		
	72h	0	0	0	0	0		
	5d	0	0	0	0	0		
" " No. 2	24h	0	0	0	0	0		
	72h	0	0	0	0	0		
	5d	0	0	0	0	0		

0 = no reaction.
± = questionable reaction.
+ = slight redness.
++ = marked redness.
+++ = slight necrosis.
++++ = ulceration.

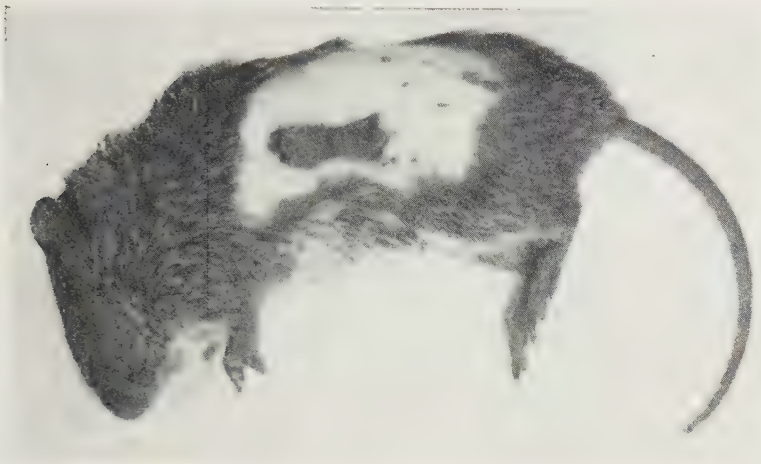

FIG. 2.
Local lesions in the cotton rat following endermal injection of 1 and 1/2 mfd of diphtheric toxin.

guinea pigs, cotton rats, and albino rats. The results are given in Table III. It will be seen that cotton rats failed to react to threshold doses of toxin, such as 1/10 and 1/50 mfd, which produce severe lesions on the guinea pig's skin. However, comparable skin-reactions, consisting of initial induration and redness followed by necrosis and ulceration, were readily obtained in cotton rats by the injection of

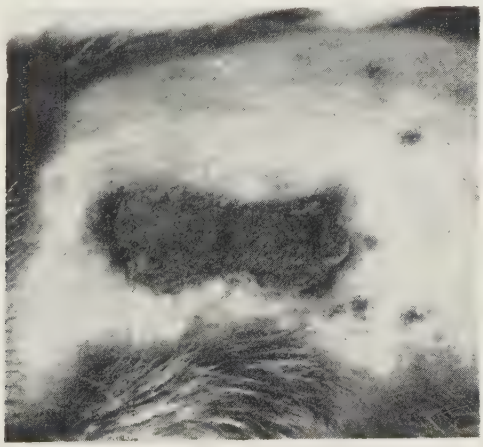

FIG. 3.
Skin-reaction with 1 and 1/2 mfd of diphtheric toxin in the cotton rat. (Fig. 2 enlarged).

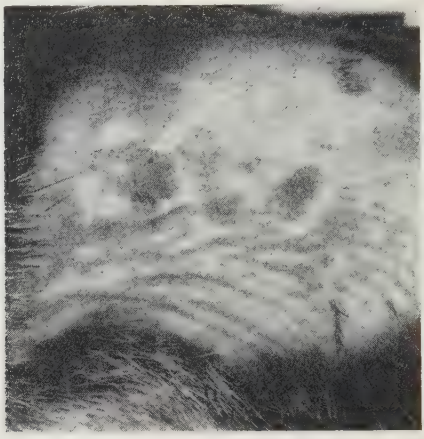

FIG. 4.
Skin-reaction with 1/5 and 1/10 mfd of diphtheric toxin in the cotton rat.

one or ½ mfd. These reactions are clearly shown in photographs 2 to 4. In keeping with previous experience, no skin-reactions were observed in albino rats with any of the doses of toxin included in our range.

Conclusions. The results reported in this paper leave no doubt that the Eastern cotton rat is markedly susceptible to diphtheric infection and intoxication. While not equalling the extraordinary susceptibility of the guinea pig, the cotton rat appears to be at least many times more susceptible than the highly resistant albino rat. The significance of this observation in relation to the zoölogical classification of the cotton rat among the group of rodents remains to be determined.

11239 P

Mucopolysaccharide Acid of Cornea and Possible Relation to the "Spreading Factor."*

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The nature of the mucopolysaccharide of the cornea has been much debated. A number of investigators have considered it to be a mucoitin sulfuric acid,[1] while others have found no sulfuric acid.[2] It was reported recently, on the basis of colorimetric analysis,[3] that the carbohydrate contained an amino sugar and sulfuric acid, but galactose instead of uronic acid. Most of the reports have been based on analyses of protein complexes ("mucoids") or of digests prepared by strong alkali.

In the past we have prepared this mucopolysaccharide acid in high yields both by alkaline digestion and by more gentle methods which avoid the use of strong alkali.[4] Our preparations contained one mol each of hexosamine, acetyl, uronic acid, and sulfuric acid, and had the same composition and general properties as those of the mucoitin-sulfuric acid obtained from gastric mucosa.[5] Good yields of glucosamine were isolated from both acids. In several important respects, however, the two compounds were found to differ: (1) the acid of cornea always formed quite viscous aqueous solutions, while that of gastric mucosa did not; (2) the specific rotation of the acid from cornea was about $-50°$, while that of the gastric mucosa was around 0 ($+2°$ to $-8°$); and (3) the acid from gastric mucosa was completely refractory to the specific enzyme from pneumococcus,[6] while the polysaccharide from cornea was hydrolyzed by this enzyme at

* This work was supported in part by a grant from the John and Mary R. Markle Foundation.

1 Levene, P. A., and López-Suárez, J., *J. Biol. Chem.*, 1918, **36**, 105; Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, 1936, **114**, 689.

2 Mörner, C. T., *Z. Physiol. Chem.*, 1893, **18**, 213; Karlberg, O., *Z. Physiol. Chem.*, 1936, **240**, 55.

3 Suzuki, M., *J. Biochem.* (Japan), 1939, **30**, 185.

4 Meyer, K., *Cold Spring Harbor Symposia on Quantitative Biology*, 1938, VI, 91.

5 Meyer, K., Smyth, E. M., and Palmer, J. W., *J. Biol. Chem.*, 1937, **119**, 73.

6 Meyer, K., Hobby, G. L., Chaffee, E., and Dawson, M. H., *J. Exp. Med.*, 1940, **71**, 137.

about half the rate of hyaluronic acid. From this latter finding and from the fact that the rotation of hyaluronic acid is similar to that of the cornea polysaccharide, we conclude that cornea polysaccharide is the naturally-occurring mono-sulfuric acid ester of hyaluronic acid.

This conclusion would seem to be significant in view of the recent report by Chain and Duthie[7] on the "spreading factor" found in testis and bacterial filtrates. According to these workers the "spreading factor" of testis by hydrolysis reduces the viscosity of synovial fluid and vitreous humor. These fluids were shown in our laboratory to contain hyaluronic acid as the viscous component, which is hydrolyzed by a specific enzyme obtained from pneumococci, streptococci, *Cl. welchii,* and spleen. The explanation of the "spreading" action in skin suggested by the work of the English authors is that the "spreading factor" acts on a "mucin" present as interfibrillar substance in the skin.

We were able to confirm the findings of Chain and Duthie and found further that testis extracts contain an enzyme which hydrolyzes pure hyaluronic acid as well as the polysaccharide acid of the cornea, while the mucoitin sulfuric acid from gastric mucosa was found to be completely refractory. The concentration of the enzyme in testis was inferior to that of the similar enzyme found in pneumococcus and group A hemolytic streptococcus. A further distinction is the pH optimum which for the bacterial enzymes was found to be 5.8 and for the testis enzyme 4.3, with hyaluronic acid as substrate.

We believe, however, that the substrate in the skin on which the "spreading factor" exerts its effect is not hyaluronic acid itself but its sulfuric acid ester. Evidence in support of this belief is furnished by the fact that such sulfuric acid esters stain meta-chromatically with Toluidine blue while hyaluronic acid does not. The substantia propria of the cornea can be stained with Toluidine blue and so also are the fibrils of the corium layer of the skin. (For a beautiful illustration of the meta-chromatic staining of the cornea, see [8].) Furthermore the protein complexes of such sulfuric acid esters are very much more stable than those of hyaluronic acid. The latter are easily brought into solution in neutral medium by dilute salt solutions; the former require concentrated salt solutions, a more alkaline reaction, and often as in the case of cornea a peptizing agent such as concentrated urea. The stability of these protein complexes is apparently

[7] Chain, E., and Duthie, E. S., *Nature*, 1939, **144**, 977.

[8] Jorpes, E., Holmgren, H., and Wilander, O., *Z. Mikrosk.-Anatom. Forsch.*, 1937, **42**, 279.

responsible for the failure of some authors to obtain the cornea polysaccharide acid. By extraction with dilute salt solution, no hyaluronic acid was extracted from cattle skin. Finally, a sulfuric acid containing polysaccharide has been obtained from skin as a protein compound giving viscous solutions by Van Lier.[9]

Since the sclera is similar chemically and histologically to the substantia propria of the cornea, except for the absence of this polysaccharide, it becomes a problem to determine whether this polysaccharide is concerned with corneal transparency.

11240

Can Strophanthin Maintain Adrenalectomized Mice?*

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In a recent report by Zwemer and Lowenstein it was suggested that adrenalectomized cats can be maintained in good condition by the administration of strophanthin.[1] This substance was administered daily in the concentration of 15 μg per kg of body weight.

We have attempted to extend these findings to the mouse. For these experiments 21-day-old mice were bilaterally adrenalectomized. A total of 140 operated animals were employed, 40 serving as uninjected controls, while the remaining 100 animals received various concentrations of strophanthin. The strophanthin was dissolved in olive oil so that the daily dose was contained in 0.1 cc of oil. Injections were made subcutaneously, beginning 24 hours after the operation and continued for 8 days.

Table I summarizes the results of the administration of strophanthin in daily concentrations of 0.02, 0.2, 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 μg, respectively. Of 40 uninjected control animals, only 4 were alive on the 10th day after adrenalectomy. The average survival of the remaining 36 animals was 4.7 days. The administration of

9 Van Lier, E. H. B., *Z. Physiol. Chem.*, 1909, **61**, 177.

* Supported in part by the Rockefeller Foundation and the Fluid Research Fund of the Yale University School of Medicine.

1 Zwemer, R. L., and Lowenstein, B. E., *Science*, 1940, **91**, 75.

TABLE I.
Survival of Adrenalectomized Mice Injected Subcutaneously with Strophanthin.

Amount per day, μg	No. of animals	No. of animals alive 10th day after adrenalectomy	Avg survival, days
0	40	4	4.7 (1-8)
0.02	15	1	3.6 (3-6)
0.2	15	1	3.8 (2-5)
2.0	15	1	4.3 (3-9)
5.0	14	1	4.2 (2-6)
10.0	14	1	4.7 (2-9)
20.0	13	0	4.5 (2-5)
50.0	7	0	2*
100.0	7	0	2*

* All the animals in these groups were dead on the 2nd day after adrenalectomy.

desoxycorticosterone acetate† in a concentration of 0.2 mg per day protected 15 of a group of 17 adrenalectomized mice.

Strophanthin proved to be ineffective in the protection of the adrenalectomized mice in any of the 8 concentrations tested. No significant difference could be noted between the strophanthin injected and uninjected animals on either of the two criteria used; that is, number of animals alive 10 days post-operative, or the average survival of those animals dying before the tenth day. Strophanthin proved to be toxic in doses of 50 and 100 μg, respectively. All the animals were dead within 24 hours after the first injection of strophanthin at these concentrations.

† I am indebted to Ciba Pharmaceutical Products, Inc., for the gift of desoxycorticosterone acetate.

11241

Chemotherapeutic Evaluation of N¹-n-Acylsulfanilylhydroxamides.

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The antienzymatic theory of the mode of action of sulfanilamide and related compounds, as specifically represented by their inactivation of catalase, is finding increasing experimental support.[1–10] Such action, however, is chiefly predicated on the formation of intermediate products of oxidation of the amino group, as represented by p-hydroxylaminophenylsulfonamide. The present study deals with a new series of sulfanilamide derivatives in which the amide group has been oxidized and the amino group blocked by acylation. In view of the fact that such blockage has destroyed *in vitro* anticatalase activity[3] and to a considerable extent the therapeutic activity of previously examined derivatives, it follows that the anticatalase activities of N⁴-n-valeryl-, N⁴-n-caproyl- and N⁴-n-heptanoylsulfanilylhydroxamide* [11] depends upon their oxidized amide groups.

Bearing in mind the fact that the hydroxamide group is already present and does not have to be formed by the bacteria, these compounds should possess powerful anticatalase and bacteriostatic properties, and because of these properties, superior therapeutic activity unless changed radically and quickly by the host. A study of the first possibility will be reported by Main, Shinn, and Mellon;[12] the study of the second is reported in the present paper.

1 Locke, A., Main, E. R., and Mellon, R. R., *J. Immunol.*, 1938, **36**, 183.

2 Locke, A., Main, E. R., and Mellon, R. R., *Science*, 1938, **88**, 620.

3 Main, E. R., Shinn, L. E., and Mellon, R. R., Proc. Soc. Exp. Biol. and Med., 1938, **39**, 272.

4 Shinn, L. E., Main, E. R., and Mellon, R. R., *Ibid.*, 1938, **39**, 591.

5 Shinn, L. E., Main, E. R., and Mellon, R. R., *Ibid.*, 1938, **39**, 640.

6 Mellon, R. R., *Modern Hospital*, 1938, October.

7 Locke, A., and Mellon, R. R., *Science*, 1939, **90**, 231.

8 Main, E. R., Shinn, L. E., and Mellon, R. R., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 115.

9 Mellon, R. R., Locke, A., and Shinn, L. E., Am. Assn. for the Advancement of Science, 1939, **11**, 98, Publication No. 11.

10 Shinn, L. E., Main, E. R., and Mellon, R. R., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 736.

11 Miller, C. C., Miller, E., and Moore, M. J., *J. Am. Chem. Soc.*, in press.

* Synthesized and donated to us by Sharp and Dohme, Glenolden, Penn.

12 Main, E. R., Shinn, L. E., and Mellon, R. R., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 593.

TABLE I.
Streptococcic (Strain C 203) Infection of Mice (1,000 Fatal Doses).

Treatment	No. of mice	No. of deaths daily during 21 days									No. of Survivors	% Survivors
		1	2	3	4	5	6	7	8	9-21		
None	75	63	7	2	2					1	None	None
Sulfanilamide	62	3	1	1		1				10	46	74
Sulfapyridine	62	1								11	50	81
N^4-n-Valerylsulfanilylhydroxamide	76	1	1		2	1		1		11	59	73
N^4-n-Caproylsulfanilylhydroxamide	79	4	2					3	3	7	60	76
N^4-n-Heptanoylsulfanilylhydroxamide	78	7	1	1		1	3		1	6	58	74

Infection: 0.5 cc of a 10^{-4} broth dilution of an 18-hour broth culture intraperitoneally (1,000 fatal doses).
Treatment: 10 mg of drug freshly suspended in 0.2 cc of 15% gum acacia orally 3 hours after infection, then once daily for 3 successive days (total 40 mg).

TABLE II.
Streptococcic (Strain Richards) Infection of Mice (1,000 Fatal Doses).

Treatment	No. of mice	No. of deaths daily during 21 days									No. of Survivors	% Survivors
		1	2	3	4	5	6	7	8	9-21		
None	20	14	5	1							None	None
Sulfanilamide	20		1		3	2	1	1		3	9	45
Sulfapyridine	20	1			1	1	2	1			14	70
N^4-n-Valerylsulfanilylhydroxamide	20		1		2		1	2	1	4	9	45
N^4-n-Caproylsulfanilylhydroxamide	39				4	3	6		4	6	16	41
N^4-n-Heptanoylsulfanilylhydroxamide	20				2	3	1	1	1	1	11	55

Infection: 0.5 cc of a 10^{-6} broth dilution of an 18-hour broth culture intraperitoneally (1,000 fatal doses).
Treatment: 10 mg of drug freshly suspended in 0.2 cc of 15% gum acacia orally 3, 22 and 46 hours after infection (total 30 mg).

TABLE III.

Type II Pneumococcic (Strain Binda) Infection of Mice (100-1,000 Fatal Doses).

Treatment	No. of mice	No. of deaths daily during 21 days									No. of Survivors	% Survivors
		1	2	3	4	5	6	7	8	9-21		
None	40	6	26	7	1						None	None
Sulfanilamide	35			4	3	3	7	6	3	3	6	17
Sulfapyridine	34		1	1			6	7	3		16	47
N^4-n-Valerylsulfanilylhydroxamide	40		2	2	4	14	10	2	1	1	4	10
N^4-n-Caproylsulfanilylhydroxamide	39			1	5	13	8	3	2	3	3	8
N^4-n-Heptanoylsulfanilylhydroxamide	39		3	2	15	13	4	2			None	None

Infection: 0.5 cc of a 10^{-6} broth dilution of an 18-hour broth culture of type II subcutaneously (100-1,000 fatal doses).

Treatment: 20 mg of drug freshly suspended in 0.2 cc of 15% gum acacia orally 3 hours after infection, then once daily for 5 successive days (total 120 mg).

Mice infected with 2 strains of hemolytic streptococci and one strain of Type II pneumococci were treated 3 hours after infection as indicated in Tables I, II, and III. Any mouse which failed to show the infecting organism in the blood or peritoneal cavity at death was excluded from the experiment. The ratio of infection to treatment was purposely adjusted so that one-half to three-quarters of the sulfanilamide- or sulfapyridine-treated mice died. In this way better comparative values are obtained than with optimum treatment. Such values may also have greater clinical significance because of the tendency of many clinicians to cut sulfonamide medication following the first signs of improvement in order to minimize objectionable side reactions.

Reference to Tables I, II and III shows that the N^4-acylsulfanilylhydroxamides are approximately equal to sulfanilamide in saving mice with hemolytic streptococcic or Type II pneumococcic sepsis.

Although the fate of the sulfonhydroxamide group cannot be followed in the body at present, all 3 compounds reduce hot Benedict reagent and cold ammonical silver nitrate readily. They also undergo deacylation to the extent that mice given 50 mg orally show approximately 10 mg % of diazotizable material, calculated as sulfanilamide, in the blood 2 hours later.

Conclusion. N^4-n-valeryl-, N^4-n-caproyl-, and N^4-n-heptanoylsulfanilylhydroxamide possess approximately the same therapeutic activity as sulfanilamide against sepsis in mice produced by 2 strains of hemolytic streptococci and one strain of Type II pneumococci.

11242 P

Effect of Sulfhydryl and Other Reducing Compounds on Decarboxylation of Pyruvic Acid by Alkaline Washed Yeast.*

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Since Grassman[1] first showed that papain and kathepsins can be activated by cysteine or by reduced glutathione many papers have appeared in the literature dealing with the influence of sulfhydryl compounds on the activity of hydrolyzing and other enzyme systems. A search of literature has not revealed any researches concerned with the effect of these compounds on carboxylase. This paper is a preliminary report on the effect of sulfhydryl and other reducing compounds on the activity of carboxylase.

The procedure used was a modification of the method of Ochoa and Peters.[2] As a source of carboxylase commercial baker's yeast which had been air-dried with the aid of an electric fan for 3-4 hours or desiccated over concentrated H_2SO_4 for several days was employed. It was freed from cocarboxylase by rapid washing of the yeast 2-3 times with M/10 Na_2HPO_4 (25-30 cc for 0.5 g yeast), and once with distilled water. The washed yeast was suspended in 5 cc M/10 phosphate buffer pH 6.2. Formation of CO_2 from pyruvic acid was measured manometrically at 28° in air with conventional Warburg manometers attached to 15 cc vessels. Each vessel contained 0.5 cc washed yeast suspension, 0.1 mg Mg as $MgSO_4$ in 0.1 cc of solution, 0.2 cc sodium pyruvate solution adjusted to pH 6.2 and containing 5 mg pyruvic acid. In each case reagents to be tested or phosphate buffer pH 6.2 were added to bring the total volume of fluid to 2 cc. The pyruvate solution was tipped into the vessel after 8-12 minutes aeration. Merck's synthetic cocarboxylase and crystalline vitamin B_1 were used throughout the experiments.†

The results of 5 experiments dealing with the activation of alkaline washed yeast by cysteine hydrochloride, reduced glutathione, sodium bisulfite and phenylhydrazine hydrochloride are listed in Table I.

* This investigation has been supported by the Christine Breon Fund for Medical Research and a donation from the California Fruit Growers Exchange.

1 Grassman, W., Dycherhoff, H., and v. Schoenebeck, O., *Z. f. Physiol. Chem.*, 1929, **186**, 183.

2 Ochoa, S., and Peters, R. A., *Biochem. J.*, 1938, **32**, 1501.

† Our thanks are due to Merck and Company for a supply of synthetic cocarboxylase and to Squibb and Company for a supply of crystalline thiamine.

TABLE I.
Effect of Sulfhydryl and Other Reducing Agents on CO_2 Production from Pyruvic Acid by Alkaline Washed Yeast.

Exp. No.	System Tested	Micro-liters CO_2 liberated
1		in 30 min
	10 γ B_1	— 11
	10 γ B_1 + 0.2 γ cocarboxylase	23
	10 γ B_1 + 2 mg cysteine HCL	130
	10 γ B_1 + 2 mg cysteine HCl + 0.2 γ cocarboxylase	177
	No B_1, 2 mg cysteine HCl	111
	No B_1, 2 mg cysteine HCl + 0.2 γ cocarboxylase	110
2		in 45 min
	10 γ B_1	3
	10 γ B_1 + 0.2 γ cocarboxylase	110
	10 γ B_1 + 0.2 γ cocarboxylase + 1 mg cysteine HCl	208
	10 γ B_1 + 0.2 γ cocarboxylase + 0.5 mg cysteine HCl	174
	10 γ B_1 + 0.2 γ cocarboxylase + 0.25 mg cysteine HCl	228
	10 γ B_1 + 0.2 γ cocarboxylase + 0.1 mg cysteine HCl	201
3		in 45 min
	10 γ B_1	— 26
	10 γ B_1 + 0.2 γ cocarboxylase	154
	10 γ B_1 + 0.5 mg GSH	82
	10 γ B_1 + 0.5 mg GSH + 0.2 γ cocarboxylase	205
	10 γ B_1 + 0.2 mg GSH	34
	10 γ B_1 + 0.2 γ cocarboxylase	230
4		in 45 min
	10 γ B_1	— 11
	10 γ B_1 + 0.2 γ cocarboxylase	76
	No B_1, no Mg	1
	No B_1, no Mg, 1 mg $NaHSO_3$	44
	10 γ B_1 + 1 mg $NaHSO_3$	49
	10 γ B_1 + 1 mg $NaHSO_3$ + 0.2 γ cocarboxylase	154
5		in 45 min
	10 γ B_1	— 15
	10 γ B_1 + 0.2 γ cocarboxylase	101
	10 γ B_1 + 0.8 mg phenylhydrazine HCl	3
	10 γ B_1 + 0.8 mg phenylhydrazine HCL + 0.2 γ cocarboxylase	151
	10 γ B_1 + 1.6 mg phenylhydrazine HCl	23
	10 γ B_1 + 1.6 mg phenylhydrazine HCl + 0.2 γ cocarboxylase	157

From an inspection of the table it can be seen that all these substances markedly stimulate the decarboxylation of pyruvic acid. Similar effects have been obtained with H_2S. These compounds were found to be effective in amounts as low as 0.01 mg for cysteine hydrochloride, 0.1 mg for glutathione, 0.02 mg for sodium bisulfite, and 0.2 mg for phenylhydrazine hydrochloride. There are 3 possible explanations for the effects observed: (1) Free SH-groups are essential for the activity of the enzyme and since all these compounds are reducing agents they may convert SS-linkages to SH-groups. (2) The compounds, particularly cysteine and reduced glutathione, may combine with the substrate to render it more active. Schubert[3] has been able to demonstrate compound formation between pyruvic acid

and SH-compounds. (3) The compounds may react with acetaldehyde, the chief reaction product; thus removing its influence. Lohman and Schuster[4] have shown that acetaldehyde inhibits the system and Schubert has observed compound formation between SH compounds and the former. The first possibility appears to us to be the most likely.

The work is being continued and will be reported in more detail at some future date. On the basis of the present work, the assay of tissues and biological fluids for cocarboxylase by the method of Ochoa and Peters[2] or Goodhart and Sinclair[5] may be open to question, since most tissues are rich in SH-compounds. This may offer an explanation for the results obtained by Lipschitz, *et al.*,[6] who found that livers of polyneuritic chicks sometimes gave values for cocarboxylase which were higher than those found for normal birds.

11243

Toxic Effect of Human Urine on Fibroblasts Growing *in vitro*.

M. RACHMILEWITZ.* (Introduced by A. Fischer.)

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It has long been held that urine possesses toxic properties. Bouchard[1] demonstrated the toxicity of urine by injecting it into the ear vein of a rabbit, determined how much was needed to kill the animal, and called quantity required per kg of body weight the urotoxic coefficient. This method came to be employed for determining the toxicity of the urine in various physiological and pathological conditions. Diminished toxicity in certain pathological conditions, especially uremia, was ascribed to the retention of toxic substances in the blood and was held to be the cause of some or all of

3 Schubert, M. P., *J. Biol. Chem.*, 1936, **114**, 341.

4 Lohman, K., and Schuster, P., *Biochem. Z.*, 1937, **294**, 188.

5 Goodhart, R. S., and Sinclair, H. M., *Biochem. J.*, 1939, **33**, 1099.

6 Lipschitz, M. A., Potter, V. R., and Elvehjem, C. A., *Biochem. J.*, 1938, **32**, 474.

* With the assistance of Miss E. Nussbaum.

Aided by a grant from the Los Angeles Fund through Dr. T. Gollia.

1 Bouchard, quoted by Volhard, *Bergmann und Staehelin's Handbuch der Inneren Medizin*, Berlin, 1931, **6**, 720.

the clinical symptoms. These experiments were the general basis for the conception of auto-intoxication.

The toxicity of the urine was later clearly demonstrated by Bruecke[2] who produced severe uremic symptoms in dogs by anastomosing the ureter of one kidney with the common iliac vein, the other kidney remaining intact. This was repeated and confirmed by Hartwich and Hessel[3] and Enderlen, Zuckschwerdt and Feucht.[4] These experiments led to the conclusion that the toxic effect of urine is not due to constituents preformed in the blood, but that the urine must contain toxins formed in the kidney.

More recently Rohdenburg and Nagy[5] have demonstrated the presence in normal human urine of growth-inhibiting as well as of growth-promoting substances, using the rate of division of the protozoon *Colpidium campylum* as an index.

A report is here given of the toxic effect of normal human urine on fibroblasts growing *in vitro*. The experiments were carried out in connection with an investigation into the toxicity of uremic blood and urine, which will be reported elsewhere.

Heart fragments of uniform size from 7-day-old chick embryos were used for explantation. After 3 passages in hanging drops they were transformed to flasks and cultivated according to the standard method of Carrel. Chick's plasma diluted with Tyrode in the proportion 1:2 coagulated by one drop of diluted embryonic extract constituted the solid phase of the media.

Normal human urine (sp. gravity 1025-1030) was taken from various healthy individuals and added in varying concentrations to the flasks; the diluent used was Tyrode or embryonic extract. The urine was taken sterile or sterilized by filtering through a Jena glass filter (5/3). The pH of the supernatant fluid of the experimental flasks with concentrated urine (0.5 cc urine diluted with the same amount of Tyrode) was practically the same as that of the control flasks; it varied from 7.6 to 7.9.

In order to establish that the hypertonicity of the concentrated urine does not interfere with growth the effect on growth rate of fibroblasts of a sodium-chloride solution of the same specific gravity as the urine (1026) was examined and the experiment showed that the cultures grow in this salt solution (in amounts equivalent to the urine) just as well as in the control flasks with Tyrode.

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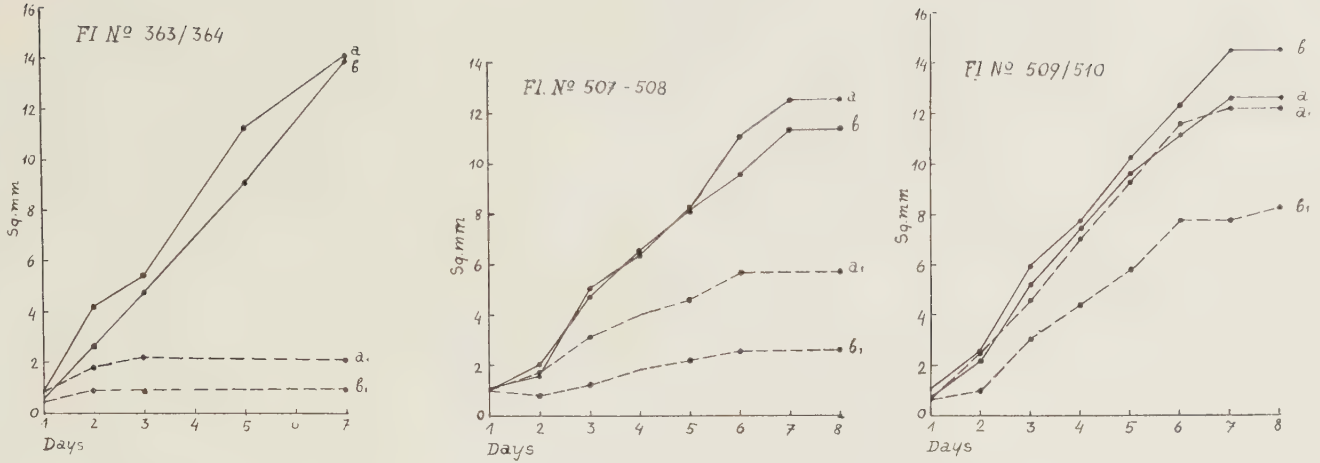
2 Bruecke, E. T., *Wien. Klin. Wschr.*, 1926, **38**, 1058.

3 Hartwich, A., and Hessel, G., *Klin. Wschr.*, 1927, **35**, 1650.

4 Enderlen, Zuckschwerdt and Feucht, *Mchner. Med. Wschr.*, 1928, **75**, 30.

5 Rohdenburg, G. L., and Nagy, S. M., *Am. J. Canc.*, 1937, **29**, 66.



a, a_1 and b, b_1—sister halves.
———— control cultures.
– – – – – – – experiment cultures.

FIG. 1.

A—growth of cultures in media containing 0.25 cc urine.
B— ” ” ” ” ” ” 0.1 ” ”
C— ” ” ” ” ” ” 0.05 ” ”

The experiments were made with sister halves of cultures, one half serving for the test, the other half for control. The cultures were observed for 5-8 days, during which period the medium was not changed. They were projected every day and drawn and measured with a planimeter in accordance with Ebelling's method. The resulting values were used in plotting the growth curves.

The experiments showed that normal human urine causes an inhibition of growth as well as degenerative changes in the fibroblasts.

The addition of 0.5 cc urine (bringing the ratio of urine to flask contents to 1:5) completely inhibits growth in the tissue fragments *in vitro*. When 0.25 cc urine (final dilution 1:10) was added, a just perceptible growth was observed (Fig. 1A). After the addition of 0.1 cc (final dilution 1:25) there was marked growth but distinctly less than in the controls (Fig. 1B). An inhibition of growth was also generally noticeable with 0.05 cc urine (end dilution 1:50), (Fig. 1C). With higher dilution of the urine up to 0.025 cc (end dilution 1:100) no difference was observed in the growth rate of the test-cultures as compared with the controls.

The colonies growing in media containing urine show not only inhibition of growth but also considerable degenerative changes in the cells. The growing zone appears dark, dense, the cells markedly granular, frequently loosely connected.

In cultures growing in media containing urine and embryonic extract, the effect of the urine was practically the same as in cultures without embryonic extract.

A further series of experiments was undertaken with a view to investigating the nature of the urinary toxic substances. So far it

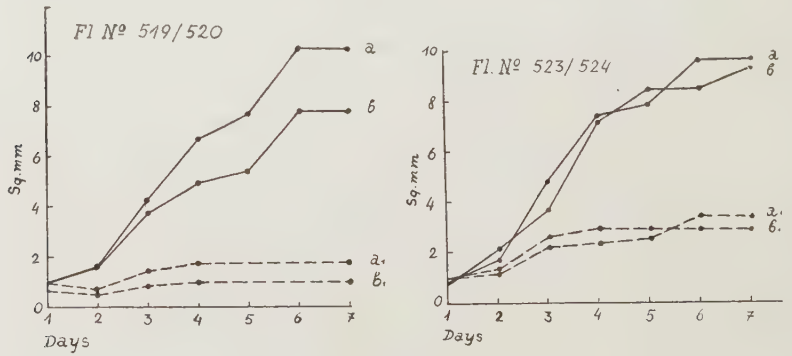


FIG. 2.

a, a_1 and b, b_1—sister halves.
———— control cultures.
– – – – – – – – experiment cultures.
A—growth of cultures in media containing 1 cc of the residue after dialysis.
B— " " " " " " 0.5 cc of the residue after dialysis.

has been shown that the toxicity of urine for fibroblasts is not destroyed by heat. Urine heated to 60°C and 100°C causes the same degree of inhibition of growth as unheated urine. It was also shown that the toxic substance in urine is non-dialysable. The addition to cultures of 1 cc or 0.5 cc of the residue after dialysis (corresponding more or less to the same volume of urine) results in a markel inhibition of growth (Fig. 2A and B), though somewhat less than is seen with whole urine.

The method described here of demonstrating the toxicity of urine by its effect on the growth rate of fibroblast colonies is thus exceedingly sensitive and enables us to measure the toxicity of urine with a great degree of exactitude. This method may therefore help in the investigation of the nature of the toxic substance present in urine as well as the toxicity of urine in various pathological conditions.

Summary. A method is described of demonstrating the toxicity of urine by its effect on the growth rate of cell colonies *in vitro*. Normal human urine in a dilution 1:5 completely inhibits the growth of fibroblasts; urine in a dilution of 1:50 still perceptibly inhibits their growth. The cells growing in media containing urine even in small concentrations exhibit signs of degeneration. Heat does not destroy this property of urine. The toxic substance is nondialysable.

11244

Effect of Concomitant Administration of Estrogens and Progesterone on Vaginal Smear in Man.*

Ephraim Shorr.

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Of the changes in the vaginal secretion during the menstrual cycle[1] those occurring in the first half concurrent with the growth and ripening of the ovarian follicle are most clearly defined and best understood. The demonstration in this laboratory[2] that they are similar to the smear changes induced in menopause and amenorrhea

* Aided by grants from the Council on Pharmacy and Chemistry of the American Medical Association and the Josiah Macy, Jr., Foundation. Grateful acknowledgment is made to Dr. Erwin Schwenk of the Schering Corporation for the estradiol benzoate (Progynon B), estradiol dipropionate (Progynon DP), progesterone (Proluton), and pregneninolone (Pranone) used in these experiments; and to E. R. Squibb and Sons for their supply of estrone (Amniotin).

by the administration of estrogens, indicates that during the first half of the cycle the vaginal epithelium and secretion are largely, if not entirely, under the control of the estrogenic hormones. The peak effect of the estrogens as seen in the fully expressed "follicular picture" induced after menopause can, therefore, serve as an index of the extent of spontaneous follicular activity during the normal cycle, and affords a simple histological measure of ovarian function. It can also be employed as a guide for replacement therapy with estrogens[2] and as a measure of ovarian stimulation by gonadotropic agents.[3]

The changes in vaginal smear during the second half of the cycle from the time of ovulation up to the next menstruation are less clear cut and more difficult to evaluate.[1] The lack of uniformity possibly arises from the more complicated hormonal pattern of this phase of the cycle. The estrogenic titer falls from its mid-menstrual peak and, except for a brief moderate rise about a week premenstrually, gradually sinks to the low levels characteristic of the premenstruum and the menstrual phase. The progestational hormone now appears for the first time, is elaborated for a period of about 10 days and then disappears one to 3 days before the flow.[4] If, as is likely, the structure of the vaginal epithelium is a resultant of the interaction of both groups of hormones, any variation in the tempo or the extent of their production could be expected to vary the cytology of the vaginal secretion.

The experiments reported in this paper are part of a study designed to analyze the influence of each of these hormonal factors on the structure of the vaginal epithelium and secretion during the second half of the cycle and to ascertain whether the vaginal smear can yield specific cytological evidence of the influence of progestin. Experiments with mice[5] and monkeys[6] have shown an antagonistic action of estrogens and progesterone on the vaginal epithelium. In the mouse, progesterone, like testosterone, prevented cornification when given simultaneously with estrone or estradiol. In the monkey, progestin (a chemically impure preparation of the corpus luteum hor-

1 Papanicolaou, G. N., *Am. J. Anat.*, 1933, **52**, No. 3, Supplement, May 15.

2 Papanicolaou, G. N., and Shorr, E., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 528; *Idem., J. Obs. and Gyn.*, 1936, **31**, 806.

3 Shorr, E., and Papanicolaou, G. N., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 629.

4 Venning, E. H., and Brown, J. S. L., *Endocrinology*, 1937, **21**, 711.

5 Robson, J. M., *J. Physiol.*, 1937, **90**, 15.

6 Hisaw, F. L., Greep, R. O., and Fevold, H. L., *Am. J. Anat.*, 1937, **61**, 487.

mone) caused a sloughing of the vaginal epithelium previously built up by estrone despite continued estrone administration.

Methods: The subjects were 9 women, 3 in spontaneous menopause and 6 with a menopausal syndrome following removal of the uterus, the ovaries, or both uterus and ovaries. The same general procedure was followed as in a previous study on the peripheral neutralization of estrogens by androgens.[7] A follicular type of vaginal smear was induced by estradiol benzoate, estradiol dipropionate, or estrone. Once this stage was reached the same dose of estrogen was continued along with progesterone, the dose of which was varied from time to time. In 2 cases pregneninolone was given by mouth. Vaginal smears were obtained daily and vaginal biopsies at appropriate times.

Results: As a basis for comparison with the changes induced by this combined therapy a brief, though necessarily incomplete, description is given of the most characteristic cytological changes noted by Papanicolaou[1] during the post-ovulatory and premenstrual phases. (1) There is usually a progressive decrease in the number of cornified cells with small pyknotic nuclei, and they may entirely disappear. (2) The discrete arrangement of the cells at ovulation is replaced by clumps of variable density. (3) The majority of the cells are of the intermediate undifferentiated squamous type, irregularly folded or with curled edges. They assume a variety of shapes including the characteristic navicular, with large, round or oval nuclei usually predominant. (4) Leukocytes usually increase in number. There is a rich bacterial growth often associated with cytolysis; and the smears have a "dirty" appearance. (5) There is an increase in mucus from time to time. (6) Cells from the deeper layers of the vaginal epithelium are occasionally seen.

Definite changes were recognizable in the smear within a short time after the institution of combined therapy. In about one-half of the cases they were detectable in 24 hours and in the remainder within 48 hours. The following changes were quite uniformly seen:

1. A leukocytosis of variable extent was present in all of the cases. The degree of leukocytosis varied irregularly, the smear occasionally becoming quite free of them for a day or two. In Case H.S. leukocytes were present in small numbers in only an occasional smear.

2. The typical cornified cells with small pyknotic nuclei rapidly diminished in number and in many instances virtually disappeared. In

7 Shorr, E., Papanicolaou, G. N., and Stimmel, B. F., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 759.

most of the smears a small number of large pale flat squamous cells with small pyknotic nuclei persisted. In Case H.S. cornified cells continued to constitute about one-fourth of the epithelial cells but most of them were folded and wrinkled.

3. Characteristic clumping took place early. The cell aggregates were usually quite dense at the end of therapy. From time to time variation in the density of the clumping was seen.

4. Superficial squamous cells of the undifferentiated type dominated the smear picture. In the earlier stages the most characteristic change was the folding of the cell. Later, curling of the edges took place and a variety of shapes and sizes of cells was seen such as the navicular, the straplike, and even the oyster-shaped cell which has been described as characteristic of pregnancy.[1] In the thick smears there was considerable compression of the cells with occasional aggregates such as are also seen in pregnancy. Cells with large nuclei increased in number, but there was a persistence and frequent predominance of cells of various shapes and sizes with small pyknotic nuclei.

5. From time to time increased mucinification was seen. Bacteria appeared frequently in great abundance. In association with this, there occurred cytolysis with large numbers of free nuclei; and the smears often assumed a smudgy appearance.

6. Although a few cells from the deeper layers of the vaginal epithelium were usually seen at the end of combined treatment they were never a prominent feature.

In the 2 cases treated with pregneninolone the changes were of a similar character to those seen with progesterone but were not as marked. Apparently higher doses of this preparation should have been used.

A detailed description of the changes in the vaginal epithelium under this regime will be given in a separate report. It will suffice here to describe the major alterations noted. The biopsies taken during the induced follicular phase showed the typical increase in the height of the epithelium as compared to the untreated state. The intra-epithelial condensation or cornification of Dierks[8] was present and the cells of the functionalis were thick walled and flattened. Following the combined administration of estrogen and progesterone there was a definite increase in the height of the epithelium contributed largely by the functionalis. The intra-epithelial zone of cornification became less prominent and in many instances disappeared. The walls of the cells of the functionalis became thinner,

[8] Dierks, K., *Arch. f. Gynaek.*, 1927, **130**, 46.

TABLE I.

Tabulation of the Dosage of Estrogen and Progesterone Administered Simultaneously to Women in Menopause.

Case	Condition	Estrogen		Estrous unit and maintenance dose R.U. per day	Progesterone mg (number days)
		Preparation			
S.B.	Natural Menopause	Estradiol	Benzoate	3,000	10 (4), 20 (6)
M.G.	" "	Estrone		1,500	10 (6), 20 (6)
A.S.	" "	"		3,500	15 (5)
T.K.	Surgical Castrate	Estradiol	Benzoate	4,000	10 (6), 20 (6), 35 (6), 100 (6)*
A.B.	" "	"	"	3,000	10 (6), 25 (5), 25 (9)
L.C.	" "	"	"	7,500	10 (7), 20 (4), 35 (5)
M.L.	" "	"	"	10,000	10 (6), 25 (7)
H.S.	Surgical—one ovary present	"	Dipropionate	5 mg 3 × week	25 (12)
M.D.	Surgical Castrate	"	Benzoate	4,000	50 (7)*, 100 (9)*

*Pregneninolone orally.

the cells wider, presenting in the most extreme cases a chicken-wire appearance. This picture is similar to that described by Dierks, and by Traut, Bloch and Kuder[9] during the proliferative phase; and in the most extreme hyperplasias, resembles the picture seen in pregnancy[9, 10] where the production of both groups of hormones is at a very high level.

Symptomatically, previous relief of the menopausal symptoms induced by estrogen persisted throughout the concomitant administration of progesterone. Occasionally increased fullness and tenderness of the breasts was noticed.

Discussion. It is apparent that the concomitant administration of progesterone and estrogen causes profound alterations in the vaginal smear as compared with the picture seen with estrin alone. Virtually all of the changes seen during the second half of the cycle are reproduced. Since the amount of estrogen was constant throughout, it was not to be expected that the changes would occur in the exact sequence seen during the normal cycle. The inference seems warranted that the changes taking place during the second half of the cycle result not only from the diminution in estrin production but also from the active influence of the progestational hormone in modifying the effect of estrin on the vaginal epithelium. This conclusion must also be true for pregnancy where both groups of hormones are present in such large amounts.

No sloughing of the epithelium as observed in the monkey was seen in this group of women although the proportion of progesterone to estrin was frequently as great as that employed in the animal experiments. The human epithelium appears to be more sensitive to progesterone than that of the mouse in that much less progesterone in proportion to estrin is effective in abolishing cornification.

[9] Traut, H. F., Bloch, P. W., and Kuder, A., *Surg. Gynec. and Obst.*, 1936, **63**, 7.
[10] Stieve, H., *Ztschr. f. mikros.-anat. Forsch.*, 1925, **3**, 307.

11245 P

Possible Relation of the Inclusion Body of Trachoma to Pathogenesis.*

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Among other reasons for regarding the causative agent of trachoma as a virus is the cytoplasmic inclusion of the epithelial cells of the conjunctiva, and occasionally even of the cornea. Marshalling together evidence that is essentially morphological, a number of investigators beginning with Halberstaedter and Prowazek[1] have attempted to prove that the inclusion represents a mass or "colony" of infectious units. While conducting a broader study† on the inclusion of trachoma and clinically allied diseases in this laboratory, observations have been made by indirect approach which are sufficiently interesting on this phase of the subject to merit their publication.[2]

In testing out the infective capacity of conjunctival tissues from different patients with trachoma, monkeys in one group were inoculated with individual tissues, all of which were found to contain inclusions. In a second group, monkeys were inoculated in a similar manner with tissues lacking inclusions. In a third group, monkeys were inoculated with pooled tissues, some of which were inclusion-bearing and others inclusion-deficient. A study of the data pertinent to these experiments reveals several interesting and suggestive results. Thus, 70 monkeys *(Macacus rhesus)* were inoculated with individual tissues from 37 patients, all containing inclusions. Typical experimental trachoma occurred in 35, or 50%, of the animals tested. On the other hand, 158 monkeys were inoculated with separate tissues from 112 patients in whom inclusions were not demonstrated. Of these animals 35, or 22%, were specifically infected. The pooled tissues which were derived from 89 patients, 44 with and 45 without inclusions, were inoculated

* Conducted under a grant from the Commonwealth Fund of New York.

† A complete report will be published in the *American Journal of Ophthalmology.*

1 Halberstaedter, L., and Prowazek, S., *Deut. med. Woch.*, 1907, **33**, 1285.

2 For a review of the subject of inclusions in trachoma, see Julianelle, L. A., *The Etiology of Trachoma*, Chap. VI, The Commonwealth Fund Division of Publications, New York, 1938.

into 106 monkeys and of these, 41, or 38%, were infected. If the figures for monkeys infected in each group are plotted in the form of a curve, the result is practically a straight line, with inclusion-bearing tissues inducing most infections, the inclusion-lacking tissues inducing least or approximately half as many infections, and the tissues pooled as indicated occupying an almost mathematically half-way position between the two extremes.

Because the above result seemed to have a certain significance, the data were reassembled to determine whether the same conclusions were approachable on some other basis. Thus, of 18 separate experiments performed with tissues containing inclusions, 13, or 72%, were considered positive (*i. e.,* animals were specifically infected). Of 42 experiments conducted with material lacking inclusions, 14, or 33%, were positive, while of 24 experiments with mixed tissues, 14, or 58%, were positive. A curve representing the data runs a remarkably parallel course with that of the preceding observations.

This is not the place to discuss the possible significance of these results. However, the implications are obvious that the presence of inclusions accompanies greatest infective capacity of trachomatous tissues, and the absence of inclusions the least, while both tissues pooled approximately on a 1:1 basis approach a value half-way between both—a result certainly to be anticipated if the pooling were to represent a simple process of dilution. While it cannot be said that the evidence is conclusive, the data are of sufficient magnitude to suggest not an accidental outcome, but an actual relation of inclusion to infectivity, even to the extent that the inclusion represents an agglomeration of virus particles. This, however, is an opinion requiring further elaboration before it can be finally accepted.

11246

Induction of Water Drive in *Triturus viridescens* With Anterior Pituitary Extract.*

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In certain well established localities the American newt, *Triturus viridescens,* shows two well-defined habitats in its life cycle. The larval and adult phases live in water while the immature phase, representing some 3 to 5 years of the cycle, lives on land. In this interesting life-cycle 2 migrations occur, that from water to land following metamorphosis and that from land to water as maturity is reached. In an attempt to determine whether or not these migrations—particularly that from land to water—are brought about by the endocrine glands Reinke and Chadwick[1] were able to induce the land phases to assume a water habitat, long before they would do so normally, by giving them intramuscular implants of pituitaries from the adults. By appropriate tests the water drive-inducing factor was shown to be a product of the anterior lobe of the pituitary. They were able to show later that this action of the pituitary occurs in the absence of both the thyroids and gonads.[2]

Is the factor which induces the water drive peculiar to Triturus, or is it a hormone distributed widely among the vertebrates but with a specific effect in Triturus? If generally present in the vertebrates, with what known pituitary principles is it to be identified? The nature of its effect in Triturus is such that one is led to believe that the same pituitary principle which is responsible for growth in the body generally also induces migration to water as maturity is reached. In order to test the presence of the water drive factor in at least one other vertebrate class and at the same time to test the growth hormone as possibly being the cause of it, an attempt has been made to induce the water drive with a commercial extract of sheep pituitary which contains the growth-promoting factor (Antuitrin G). A total of 49 land phases (red efts) of Triturus were injected and set up for observation according to procedures previously described.[2]

* Antuitrin G, Parke Davis.

1 Reinke, E. E., and Chadwick, C. S., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 691.

2 Reinke, E. E., and Chadwick, C. S., *J. Exp. Zool.*, 1940, **83**, 223.

A preliminary test was made on 18 efts. Six, ranging from 60 to 90 mm in length and given 3 injections of 1 RU each at 48-hour intervals, all entered water within 122 hours. Six, ranging from 83 to 95 mm in length and given 3 injections of 1 RU each at 24-hour intervals, entered water within 92 hours. Six 78 to 93 mm efts, each given 4 RU in 2 equal amounts 24 hours apart, entered water in 72 to 100 hours. All 18 efts molted by the 3rd day following the initial injection and all had entered water by the 5th day. Their adaptation to water was apparently complete and by the 10th day their color had undergone a profound change from the red of the land phase to the green and yellow of the adult water stage. Controls, either uninjected or injected with tap water or Antuitrin S† did not enter water nor did they molt nor show any change in color.

In an attempt to determine the threshold dosage of the extract necessary to induce the water drive. 12 efts ranging from 50 to 90 mm in length, were each given single injections varying from 0.3 to 2 RU. Eleven of the 12 entered water at intervals of 3 to 10 days. Only 2, each receiving 2 RU doses, remained permanently adapted. The others were in and out of water rather indifferently and, when it appeared that there could be no correlation between dosage and time required to enter water to stay, each eft was given enough additional extract to make a total dosage of 3 units. All of these efts molted and, with the exception of the one 50 mm eft, were well adapted to water when killed 2 to 3 weeks after the initial injection. These observations indicate that 2 to 3 RU of Antuitrin G are necessary to induce the water drive, providing the injected animals are above 50 mm in length.

In order to check further the failure of the extract to induce small efts to go to water, 7 efts, ranging from 46 to 55 mm in length, were each given 6 half-unit injections at 12-hour intervals. Although all molted on the 2nd and 3rd days and entered water by the 6th day, none of them remained consistently in water during the 3-week period of observation. Additional injections of the extract failed to make them become completely adapted to water.

Since Antuitrin G contains both the gonadotropic and thyrotropic anterior pituitary factors, it was considered of interest to see if the extract would induce the water drive in efts deprived of their gonads and thyroids. Six gonadectomized efts ranging from 75 to 90 mm in length and given 3 RU in 3 days, responded identically with the animals in the preliminary test. Only 1 of 6 thyroidectomized efts

† Parke Davis Co. This extract contains the anterior-pituitary like factor and had been shown previously not to be effective in inducing the water drive.

of similar size and given the same treatment entered water and none molted.

To summarize briefly, 35 large efts ranging from 60 to 95 mm in length, either normal or gonadectomized, were driven to water within 5 days by injections of 2 to 3 RU of mammalian pituitary extract. Similar quantities failed to cause 8 efts less than 55 mm in length and 5 of 6 large thyroidectomized efts to enter water.

While mammalian pituitary extract does not consistently induce the water drive in small efts nor in efts deprived of their thyroids, the fact that it invariably causes large normal or gonadectomized efts to migrate to water is proof that the water-drive factor is not restricted to Triturus alone. Although the return of Triturus to water is necessary for reproduction, the gonads seem not to be concerned in any way with the initiation of the water-drive. The failure to induce the water drive consistently in thyroidectomized efts by injection of mammalian extracts when implants of adult Triturus pituitary readily do so[2] indicates either a species difference or that some qualitative change has been brought about in the mammalian factor due to the manner of extraction. The failure to induce the drive in small efts is also quite interesting but before any conclusions can be drawn as to these failures a greater series of thyroidectomized and small efts must be injected. The acceleration of the acquisition of adult characteristics along with the water drive in large efts by injection and in efts of any size by implantation of adult Triturus pituitary together with the fact that the water drive is induced by a recognized growth hormone-containing extract, indicates that the growth hormone is also the water-drive hormone.

11247

The Bacteria-Free Culture of a Nematode Parasite.

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It has been possible for some time to grow the entire life cycle of *Neoaplectana glaseri,*[1] a nematode parasite of the Japanese beetle, in cultures in which bacterial and fungous growths have been inhibited in various ways but not eliminated.[2] These contaminants undoubtedly introduced a high degree of variability into the results obtained. It therefore seemed advisable to attempt to rear this parasite in cultures free from bacteria.

Lapage[3] and Glaser and Stoll[4] developed technics whereby the second ecdysis of strongyloid nematode larvae was easily and consistently obtained in quantity under sterile conditions. It was found necessary to modify one of these technics slightly for work with *Neoaplectana.* Cultures prepared in the routine manner were permitted to develop for 10 to 15 days, at which time the majority of the parasites were second-stage larval forms in their third or fourth generation.* These were removed from the surface of the solid medium and washed with water until free of much débris. To remove the dead larvae they were then filtered through 2 layers of lens paper supported by fine gauze. The larvae were ensheathed by aerating them in 25 cc of water for 3 to 4 days with a change of clean water 3 times daily. They were then washed in 25 cc lots of sterile water 3 times each day for 3 days, after which they were treated for 30 minutes to 1 hour with Labarraque's solution (sodium hypochlorite) at a dilution of 1 part to 40 or 50 parts of water. The nemas were again washed 3 times in sterile water, and then placed in

1 Steiner, G., *J. Washington Acad. Sci.*, 1929, **19**, 436.

2 Glaser, R. W., *Science*, 1931, **73**, 614; Circ. 211, Dept. of Agric., State of New Jersey, 1932; *Studies from The Rockefeller Institute for Medical Research*, 1932, **83**, 521; McCoy, E. E., and Glaser, R. W., Circ. 265, Dept. of Agric., State of New Jersey, 1936; McCoy, E. E., and Girth, H. B., Circ. 285, Dept. of Agric., State of New Jersey, 1938.

3 Lapage, G., *J. Helm.*, 1935, **13**, 103; *Parasitology*, 1935, **27**, 186.

4 Glaser, R. W., and Stoll, Norman R., *J. Parasit.*, 1940, **26**, 87.

* The ensheathed second-stage larva is the only stage capable of surviving free in nature and represents the invasive form which must penetrate into a host (Japanese beetle grub) to continue its development.

water about 5 mm deep for from 15 to 20 hours.† The next day the nemas were again treated with Labarraque's solution and then washed 3 times, followed by another 15 to 20 hours' sojourn in a small amount of water. This was followed by a third treatment with Labarraque's solution and 3 more washings with sterile water. Finally, the nemas were again passed through sterile lens paper, to remove any worms that had died during the manipulations, and the viable forms were stored in shallow water until used.

When the above procedures were carefully followed sterile larvae were obtained, shown by the fact that no bacterial growth occurred when they were cultured on standard laboratory media under both aerobic and anaerobic conditions. When occasional contaminants appeared later after a prolonged incubation period, such cultures were either placed aside for collateral observations or discarded.

At first it was thought necessary to have a solid substrate to facilitate the movements and ecdyses of *Neoaplectana.* Neutral veal infusion agar slants may be used, but a simple substrate of 2% agar prepared with 0.5% sodium chloride solution answered just as well. Ten cc of the melted agar were slanted in culture tubes measuring 180 x 22 mm. About one gram of animal tissue, removed under sterile conditions, was then placed at the base of the slant, and 2 or 3 drops of sterile 0.5% salt solution were added. Each tube was inoculated with ± 200 of the previously sterilized second-stage larvae and then sealed by pouring melted sealing wax over the cotton stopper previously trimmed and pushed down into the tube for approximately half an inch. When hard the sealing wax was perforated by a hot wire. Sealing in this manner prevents excessive evaporation without excluding oxygen. Recently we have also sealed many tubes with "Parafilm" perforated with a few needle pricks and have found this satisfactory. The sealed tubes were held in a slanted position and incubated at room temperatures, 22-28°C.

Eighteen- to 20-day-old mouse embryo, beef kidney, and rabbit ovary and kidney have all been found to support growth. The last proved to be the easiest to manipulate and to give the best growth. Consequently it has been used for most of the work. In tubes containing approximately one gram of rabbit kidney the tissue is almost completely digested in from 18 to 24 days; at this same period of incubation, growth has reached its maximum and second-stage larvae predominate. Cultures may be held for at least 3 months without transplantation, but to maintain vigorous growth, transfers are made

† During this time interval *Neoaplectana* digested and evacuated the greater part of its intestinal flora.

every 3 weeks. Sterile nematodes have to date been maintained without loss through 14 transfers or approximately 50 generations. With the methods previously used it was necessary to add certain accessory growth factors in order to maintain the cultures longer than 21 to 32 generations.[5] The nematodes have also been cultured in Erlenmeyer flasks containing 0.5% NaCl solution to a depth of 3 to 4 mm in which are placed pieces of sterile rabbit kidney, and in liquid media containing kidney extracts devoid of particulate matter.

On agar slants containing rabbit kidney the number of nematodes found after incubation for 18 days is approximately 150,000. When grown on Petri dishes, 5.5 cm in diameter, containing 2% dextrose agar and living yeast, the yield was about 40,000. Contamination of the rabbit kidney causes a marked reduction, and in some cases an absence, of growth.

In view of the fact that this parasitic nematode can be grown on both liquid and solid media free from bacteria it will be possible to study its nutritional and other requirements and to test the effects of various vitamins and hormones.

11248 P

Effect of Desoxycorticosterone on Plasma Volume in Intestinal Obstruction.

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Adrenal cortical hormone restores the reduced blood volume in Addison's disease[1, 2] and prevents the drop in plasma volume said to occur during ether anesthesia.[3, 4] This communication reports some experimental observations on the effect of desoxycorticosterone

5 Glaser, R. W., *J. Exp. Zool.*, in press.

1 Thorn, G. W., Howard R. P., and Emerson, K., Jr., *J. Clin. Invest.*, 1939, **18**, 449.

2 Loeb, R. F., Atchley, O. W., Ferrebee, J. W., and Ragen, C., *Trans. Assn. Am. Phys.*, 1939, **54**, 285.

3 McAllister, F., and Thorn, G. W., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 736.

4 Ragan, C., Ferrebee, J. W., and Fish, G. W., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 712.

acetate* on the fall in plasma volume which occurs in acute intestinal obstruction in dogs.[5]

The dose of hormone used was arbitrarily determined. One or 1½ mg per day is necessary to sustain an adrenalectomized dog.[6] Since we were dealing with a rapidly exhausting state in our experiments we injected 5 mg at the beginning of the experiment and repeated the dose once or twice at 4-hour intervals.

Results. In Table I it will be observed that in dogs not receiving hormone the longer the distention of the small intestine continued, the greater was the loss of plasma. This averaged 35% after 4-6 hours and 55% after 18-23 hours. The detailed data of these experiments, already published,[5] show no great disparity between individual measurements and the average figure.

Of the 4 dogs which received desoxycorticosterone the first lost 38% of the plasma in the first 6 hours. There was, however, no significant change in the following 6 hours. The final measurement showed an actual return of a large part of the lost plasma to the circulating blood—an observation never observed in continuously distended dogs not receiving hormone. The first dose of hormone in this dog was given intramuscularly, while all other doses in this and the other 3 dogs were given intravenously. In the second dog of this group there was an increase in the loss of plasma from 9%

TABLE I.
Distention of Small Intestine.
% Change in Plasma Volume.

	After 4-6 hr	8-12 hr	18-23 hr
D	—36 (9)	—43 (2)	—55 (5)
D (Hormone)	—38	—40	—16
	— 9	—21	—
	—	+49	+24
	— 5	+ 5	—
C	— 8 (7)	—	—12 (7)

D—Distended dogs.
D (Hormone)—Distended dogs receiving desoxycorticosterone.
C—Control dogs without distention or with distention of other hollow organs.
Figures in parentheses represent the number of separate observations, the results of which were averaged to obtain the accompanying figures. The data of the D (Distended) dogs has already been published[5] (Table I, Group 3). The individual results are sufficiently close to justify using the average result. The data of the C (Control) dogs is partly from the same published data[5] (Table I, Groups 1 and 2) and from data to be published, in which the individual results are likewise sufficiently uniform to permit use of the average result. The hollow organs distended without marked loss of plasma were the colon and the gall bladder.

* We are indebted to Ciba & Co. who supplied this material.

5 Gendel, S., and Fine, J., *Annals Surg.*, 1939, **110**, 25.

6 Thorn, G. W., Engel, L. L., and Eisenberg, H., *Bull. J. H. H.*, 1939, **64**, 155.

after the first 6 hours to 21% after 12 hours. These figures are of a much lower magnitude than was observed in the dogs not receiving hormone and to this extent the hormone may be considered to have exerted a protective influence. In the third dog an astonishing increase in plasma above normal was noted in the 8-12-hour period of distention, and though not sustained in the subsequent interval before death occurred, a substantial positive effect seems to have been maintained by the hormone. The measurements of plasma loss in the fourth dog also show the capacity of the hormone to prevent a loss of plasma. The effect of desoxycorticosterone is therefore one of counterbalancing the action of the agent responsible for the loss of plasma.

The possible influence of other factors on the plasma volume changes are excluded by the results of a group of 7 control experiments which included 2 intact dogs under nembutal anesthesia, 2 dogs with obstruction but no distention, 1 dog with distention of the gall bladder, and 2 with distention of the colon. None of these animals showed any marked loss of plasma at any time. The observations in the last 3 dogs of this series, which suggest that the plasma loss from distention of a hollow viscus is more or less specific for the small intestine, will be published elsewhere in more detail.

The apparently favorable response to the trauma of distention in the 4 dogs receiving hormone applies, so far as our data permit a judgment, only to the plasma volume. There was no prolongation of life by the hormone. This may be due to the undesirable effects of overdosage.[2]

Conclusion. The marked fall in plasma volume observed in dogs subjected to continuous distention of the small intestine is at least partly prevented by the intravenous administration of desoxycorticosterone.

11249 P

Cuta[illegible]-Visceral Vasomotor Reflexes in the Cat.

Albert Kuntz and L. Anson Haselwood.

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The use of localized cutaneous stimulation in the treatment of visceral and other deeply located lesions has been based mainly on empirical considerations. Certain investigators, particularly Boas[1] and Freude and Ruhmann,[2] advanced data which support the assumption that localized cutaneous stimulation by means of warm applications results in vasodilatation in the corresponding segments of the gastro-intestinal tract. Certain data also support the assumption that localized cooling of the skin results in vasoconstriction in the viscera in the corresponding segments. There is no general agreement regarding the vascular reactions in the viscera elicited by localized cutaneous stimulation or the mechanisms through which such reactions are brought about.

The present series of experiments has been carried out to determine more accurately than has been indicated by previous studies whether appreciable circulatory changes in visceral organs can be brought about by loocalized cutaneous stimulation and whether the changes which occur represent direct effects of the stimulation employed or reflex phenomena.

Decerebrate preparations of the cat have been used in order to avoid the vitiating effects of anesthesia. The stimulating agents employed have been warm and cold applications and vacuum cups applied to the skin of the back and lateral surfaces of the trunk from which the hair had been removed. The circulatory changes brought about in the stomach and intestine were observed with the viscus exposed through a midventral incision, and recorded by means of photography and plethysmograph records.

Moderate cooling of the skin of the back or lateral surface by means of cold applications from the fifth or sixth thoracic segment caudalward consistently resulted in vasoconstriction in the stomach and intestine. Moderate warming of the skin in the same areas by means of warm applications at approximately 45°C consistently resulted in vasodilatation in the stomach and intestine. (Fig. 1.) Stimulation of the skin by means of vacuum cups resulted in

1 Boas, I., *Deutsch. med. Wchschr.*, 1926, **52**, 349.

2 Freude, E., and Ruhmann, W., *Z. f. d. ges. exp. Med.*, 1926, **52**, 338.

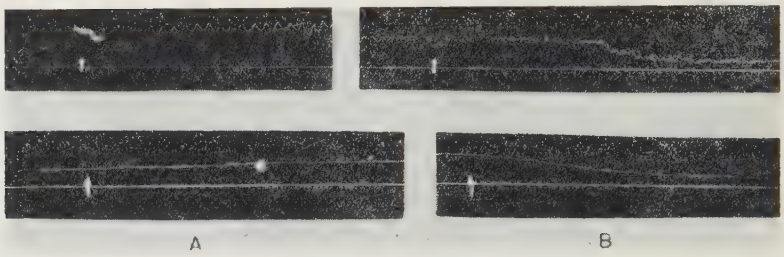

FIG. 1.

Plethysmograph records of a loop of the small intestine of the decerebrate cat during localized cutaneous stimulation by means of warm and cold applications. Initiation of stimulation is indicated by arrows.

A, Records of responses to warm applications.

B, Records of responses to cold applications.

vasodilatation in the corresponding portion of the gastrointestinal tract in approximately the same degree as moderate warming of the skin in the same area.

Photographs of segments of the gastrointestinal tract obtained during intervals of vasodilatation due to local warming of the skin and during intervals of local cooling of the skin, compared with those obtained in the absence of specific cutaneous stimulation, indicate that the changes in calibre elicited by means of the stimulation employed are more marked in the smaller blood vessels than in the larger ones. Direct observations under low magnification also support this conclusion. The changes observed and recorded are of sufficient magnitude to warrant the conclusion that the volume of blood circulating through the affected segments of the gastrointestinal tract is markedly increased by local warming and markedly decreased by local cooling of the skin.

The circulatory changes in the gastrointestinal tract observed in these experiments cannot be explained on the assumption that the gastrointestinal blood vessels were influenced directly by the stimulation employed, but must be regarded as reflex responses brought about through segmental and intersegmental reflex arcs including sympathetic neurons. The assumption that the sympathetic nerves in question include vasodilator fibers is supported by conclusive experimental data (Burn).[3]

Stimulation of the receptors involved in cutaneo-visceral vasomotor reflexes probably is associated with changes in the tonic state of the cutaneous blood vessels. In our experiments, prolonged application of warm packs resulted in localized cutaneous hyperemia which persisted for some time after removal of the stimulating agent.

[3] Burn, J. H., *Physiol. Rev.*, 1938, **18**, 137.

During this interval cooling of the skin in the hyperemic area did not result in vasoconstriction in the gastrointestinal tract, nor did further application of a warm pack result in increased vasodilatation in the viscus. This is in full accord with the observation reported by Ruhmann[4] that local warming of the skin does not elicit reflex responses of the gastrointestinal musculature until dilatation of the cutaneous vessels in the stimulated area has taken place.

11250

Response of Gonads and Gonaducts of Ambystoma Larvae to Treatment with Sex Hormones.*

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For many years Ambystoma has served as an experimental animal in studies of sex differentiation and sex development. Such studies have been made principally by means of parabiotic union of larvae or by gonad transplants. Since synthesized crystalline sex hormones are now available it seems of interest to determine the effects of these compounds upon the sexual development of the same species of salamanders and to compare the results of such experiments with those obtained by the above mentioned methods.

First reports on effects of crystalline sex hormones on sex differentiation in Ambystoma were made by Burns,[1, 2] who concluded that injection of testosterone propionate into *Ambystoma punctatum* larvae causes genetic females to differentiate in a male direction, while estrone causes differentiation of genetic males in a female direction. Ackart and Leavy[3] obtained results similar to those of Burns with injections of estrone into *Ambystoma tigrinum* larvae.

The animals used in our study were larvae of *Ambystoma maculatum* of two races, a differentiated race from Georgia, and a semi-differentiated race from Arkansas, and a small lot of *Ambystoma tigrinum* larvae from Iowa. The sex hormones used were estrone (Theelin: Parke-Davis Co.), estradiol dipropionate (Diovocylin:

4 Ruhmann, W., *München. med. Wchschr.*, 1933, **80**, 17.

* Aided by grants from the National Research Council, Committee for Research in Problems of Sex.

1 Burns, R. K., *Anat. Rec.*, 1938, **71**, 447.

2 Burns, R. K., *Anat. Rec.*, 1939, **73**, 73.

3 Ackart, R. J., and Leavy, S., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 720.

Ciba), and testosterone propionate (Perandren: Ciba).† These hormones, dissolved in absolute ethyl alcohol, were added daily to aquarium water in concentrations of 500 gamma per liter. The entire lot of animals was divided into three groups, one to receive testosterone treatment, a second to be treated with estrogens, and a third to serve as controls.

The larvae of *Ambystoma maculatum* (Georgia) of differentiated race received hormonal treatment between the ages of 70 and 142 days. *Ambystoma maculatum* (Arkansas) larvae of semi-differentiated race were treated when between 57 and 110 days old. Hormonal treatment was stopped when these animals metamorphosed. The tiger salamanders received treatment from the 50th day to the 221st day, the treatment being continued 70 to 100 days after metamorphosis. At the time of first administration of hormone there were 50 control larvae, 70 animals to be treated with testosterone propionate, and 70 animals to receive estrogens. Animals which died before the end of the experiment are included in data given in Table I.

TABLE I.

Treatment	Females	Hermaphrodites	Males with cortex	Males
Controls	27	8	6	12
Testosterone	34	8	19	7
Estrogens	53	13	2	0

Some of the control males retain a vestige of cortex but otherwise possess typical testicular structure. Some gonads of the larvae in the Arkansas group are of distinctly hermaphroditic character but must still be considered as genetic males (Witschi[4]). The gonoducts in control animals show no evidence of stimulation (Fig. 1). The Wolffian ducts are developed to their full length in all larvae. In *Ambystoma maculatum* only the anterior portion of the Mullerian ducts is present at the time of metamorphosis, but in the older *A. tigrinum* animals they reach the cloaca. These ducts are of the neutral juvenal type as described by Rodgers and Risley.[5] In no cases were the cloacal glands stimulated.

The sex ratio in the group of larvae receiving testosterone propionate treatment remains unaltered (34 females and 34 males). This seems to indicate that the male hormone has little, if any, effect upon sex differentiation. The ovaries seem to have been affected by the treatment in that there is no indication of the presence of an

† We wish to express our thanks to Ciba Pharmaceutical Products, Inc., and to Parke-Davis Co. for the generous supply of sex hormones.

4 Witschi, Emil, *J. Exp. Zool.*, 1933, **65**, 215.

5 Rodgers, L. T., and Risley, P. L., *J. Morph.*, 1938, **63**, 119.

ovarial cavity (Fig. 2). In male sex glands one observes paradoxical feminization effects which will be discussed in a forthcoming paper. In all animals of this group, regardless of genetic sex, there is an extreme stimulation of the Wolffian duct (Fig. 2) and the cloacal glands. The Wolffian and pronephric ducts are much convoluted, with crypts and pouches protruding from their walls, and in most cases the ducts are deeply pigmented and extend the full length of the body cavity. The Mullerian ducts cannot be seen in the testosterone-treated animals. This picture offered by the ducts is unequivocal proof that the testosterone is actually taken up from the water and produces characteristic effects even at very early stages of larval development.

In estrogen-treated animals the sex ratio is definitely shifted in the female direction, with 53 females and 15 males. These 15 "males", none of which possessed typical testes, are all found among the larvae which died relatively early. They have hermaphroditic gonads with testicular and ovarial features of various proportions. The ovaries of the 53 females resemble those of controls (Fig. 3). It is statistically evident that these "females" are of two genetical types, true females and sex reversed males, though morphologically they cannot be separated. While in metamorphosed animals of control groups the oviducts extend through the full length of the body cavity down to the cloaca, in estrogen-treated animals of same age they often end blindly before reaching the cloaca. This inhibition in longitudinal growth is in contrast to the considerable inflation and consequent enlargement in diameter of these oviducts (Fig. 3). At the larval stage only the upper parts of the oviducts, growing down from the pronephric region, are present and exposed to hormonic influences. These cranial parts are moderately enlarged in most cases. The Wolffian ducts and cloacal glands retain a condition as in animals receiving no hormonal treatment. The appearance of the oviducts is sufficient proof that, like the testosterone, the estrogenic hormones are also taken up by the larvae.

Conclusions. The presented data indicate that estrogens produce sex reversal in male salamanders, while testosterone propionate exerts no corresponding influence upon genetical females. As in adult animals, the secondary sex characters respond also in the larval salamanders to the administration of sex hormones. The female hormone stimulates slightly the larval Mullerian ducts, and the male hormone causes an extensive and very precocious stimulation of the larval Wolffian ducts and cloacal glands. It is remarkable that the estrogens affect most profoundly the male gonads and the testosterone the male secondary sex characters. The hormones do not induce

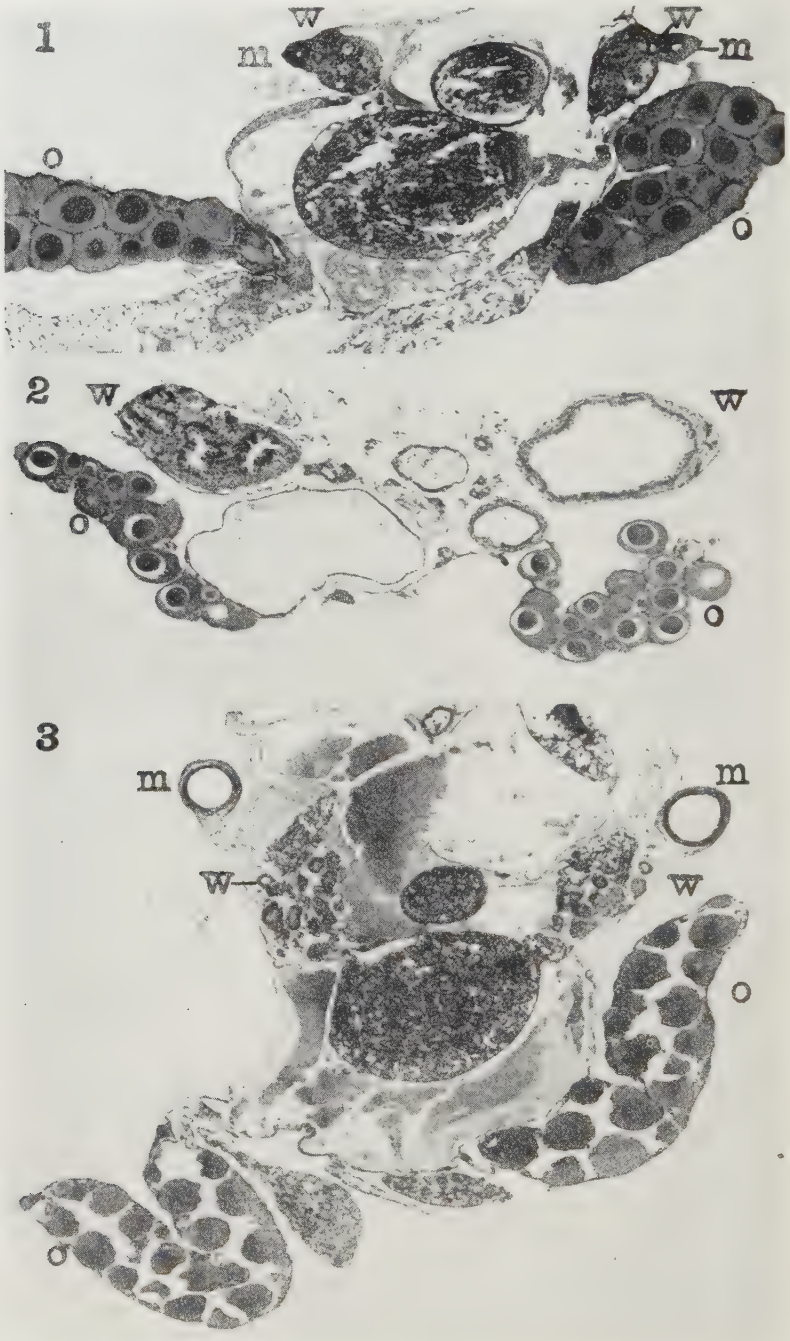
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FIG. 1.
Cross section through the gonads and gonoducts of *A. tigrinum* control female. Note unstimulated Mullerian ducts (m) and Wolffian ducts (w).

FIG. 2.
Cross section through the gonads and gonoducts of *A. tigrinum* female treated with testosterone propionate for 171 days. Note absence of Mullerian ducts and stimulated Wolffian ducts (w) and close resemblance of the right duct (left in the picture) to the frog seminal vesicle.

FIG. 3.
Cross section through the gonads and gonoducts of *A. tigrinum* female treated with estradiol dipropionate and theelin for 171 days. Note stimulated Mullerian ducts (m) and unstimulated Wolffian ducts (w). All figures ×25.

original formation of gonoducts; they stimulate only the secondary (functional) enlargement of parts of ducts already present. It has been shown by means of parabiosis that the testes of males release some inductive substance which inhibits the development of the ovaries of female cotwins and indirectly may cause some genetic females to continue development in a male direction.[6] On the other hand, in the male-female parabiotic combinations there is no precocious stimulation of either gonoducts or cloacal glands. These fundamental differences in the observed reactions prove that the crystalline sex hormones used in this experiment cannot be identical with the substances which normally act as inductors of sex differentiation.

11251

Effect of Foods on Serum Esterase of Rats.

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In a previous publication[1] it was reported that the tributyrin splitting property (here called esterase concentration) of the serum of rats is markedly increased after poisoning with carbon tetrachloride and after feeding a high fat diet. On the other hand, substances such as xanthine, which exert a protective action on the liver against carbon tetrachloride, will, when subcutaneously injected into rats, definitely lower the serum esterase concentration. Before proceeding further with the investigation into the possible significance of the serum esterase change in the animal's resistance to carbon tetra-

6 Witschi, Emil, *Allen's Sex and Internal Secretions*, Baltimore, 1939, 2nd ed., 145.

1 Forbes, J. C., and Outhouse, E. L., *J. Pharm. and Exp. Therap.*, 1940, **68**, 185.

chloride, it was necessary to study in greater detail the effect of various foods on the esterase concentration, laying particular emphasis on the changes resulting from the administration of single doses of fatty substances. In our previous recorded experiments the animals were kept on the high fat diet for a number of days and were allowed food up to the time of sacrificing.

The results herein presented deal with the effect of single doses of various foods on previously starved animals. They show that non-lipoid food stuffs, sucrose, glycerol and proteose-peptone exert no demonstrable effect on the serum esterase while neutral fats or fatty acids markedly increase it. No significant change was obtained in any case in the neutral fat, phospholipid or cholesterol content of the liver.

Experimental. Rats weighing approximately 200 g, after being starved for the previous 24 hours, were given the various supplements by stomach tube, except in the case of the Purina and Purina plus 40% butter fat diets. In these cases the required amount of food was fed early in the morning and only animals which consumed practically the whole amount in at least an hour and one-half were used for the various studies. The serum esterase, liver neutral fat, total phospholipid and cholesterol were determined by the methods previously described.[1, 2] The experimental results are shown in Tables I, II, and III.

It will be seen from the results recorded in Tables I, II and III that the administration of non-lipoid foods does not measurably affect the tributyrin-hydrolyzing power of the rat's serum. Glucose has also been used and found to be inactive. The administration of fats, oleic and palmitic acids, on the other hand, resulted in a marked rise in the serum esterase concentration. Since glycerol is inactive, while fatty acids have a pronounced effect, it would appear that the increase in esterase value is in some way related to an increase in some phase of fatty acid metabolism. The lack of apparent response following the feeding of Purina dog chow itself, which contains approximately 4% of ether extractable materials, indicates that a fair amount of fatty acids must be absorbed in order to obtain a definite response by the analytical method employed.

In a previous publication[1] it was shown that the serum esterase concentration of rats fed a high fat-low choline diet for several weeks, at which time a high fat content of the liver was produced, did not apparently differ from that obtained by feeding a high fat-Purina dog chow diet on which the deposition of fat in the liver was quite moderate. Other experiments in which sufficient choline was added to a high fat-low choline diet to prevent fat deposition in the

[2] Outhouse, E. L., and Forbes, J. C., *J. Lab. and Clin. Med.*, in press.

TABLE I.

Effect of Feeding Fat and Oleic Acid on the Serum Esterase and Liver Lipids of Rats.

No. of rats	Serum esterase			Liver, %			Remarks
	Max.	Min.	Avg	N.F.	P.L.	Chol.	
9	25	14	20	0.9	4.13	0.25	Controls.
4	31	17	25	2.0	4.06	0.28	Killed 1 hr after feeding by stomach tube 1 cc of butter fat per 100 g.
5	34	18	30	1.5	4.19	0.27	" 2 " " " as above.
2	33	30	31	1.0	4.06	0.29	" 3 " " " " "
5	36	28	33	1.2	4.18	0.26	" 4 " " " " "
2	25	20	22	0.8	3.83	0.25	" 5 " " " " "
5	28	21	25	2.0	4.23	0.27	" 6 " " " " "
2	24	22	23	1.1	3.78	0.28	" 7 " " " " "
2	23	21	22	2.2	4.36	0.27	" 8 " " " " "
1	—	—	19	1.1	4.77	0.24	Control.
2	26	23	24	1.0	4.45	0.26	Killed 2 hr after feeding by stomach tube 1 cc of olive oil per 100 g.
2	29	26	27	1.2	4.79	0.29	" 4 " " " as above.
2	37	22	34	1.7	4.78	0.29	" 6 " " " " "
2	37	26	31	1.7	4.58	0.25	" 8 " " " " "
2	28	26	27	—	—	—	" 10 hr 30 min after feeding as above.
1	—	—	22	1.3	4.58	0.26	Control.
1	—	—	26	1.6	4.38	0.26	Killed 1 hr after feeding by stomach tube 1 cc of oleic acid per 100 g.
3	33	28	30	1.2	4.35	0.27	" 2 " " " as above.
3	36	34	35	1.7	4.24	0.25	" 4 " " " " "
3	44	38	41	1.4	4.29	0.25	" 6 " " " " "
2	35	28	31	—	—	—	Killed 3 hr 20 min after feeding by stomach tube 0.4 g sodium palmitate per 100 g.

N.F. = Neutral fat plus cholesterol.
P.L. = Total phospholipids.

TABLE II.

Effect of Purina Dog Chow, and 60% Purina Plus 40% Butter Fat on the Serum Esterase and Liver Lipids of Rats.

No. of rats	Serum esterase			Liver, %			Remarks
	Max.	Min.	Avg	N.F.	P.L.	Chol.	
4	24	15	19	1.5	3.89	0.29	Controls.
5	24	15	19	1.2	3.89	0.28	Killed 2 hr 30 min after feeding 5 g Purina.
5	23	15	18	1.1	3.58	0.27	" 4 " 30 " " " as above
5	24	13	18	1.3	3.73	0.27	" 6 " 30 " " " " "
3	16	16	16	1.4	3.76	0.25	" 8 " after feeding as above.
4	22	17	19	0.9	3.78	0.29	Controls.
2	34	32	33	1.1	3.85	0.31	Killed 2 hr 45 min after feeding 3 g of 60% Purina + 40% butter fat.
2	32	32	32	1.4	3.18	0.25	Killed 4 hr after feeding as above.
2	20	16	18	0.8	3.75	0.26	" 5 " " " " "
4	24	14	20	1.6	3.37	0.27	" 7 " " " " "
4	29	18	25	1.5	3.50	0.23	" 9 " " " " "
2	20	10	15	2.2	3.16	0.25	" 25 " " " " "
4	24	21	22	1.1	3.74	0.36	Controls.
4	38	26	31	1.3	3.50	0.37	Killed 2 hr 45 min after feeding 5 g of 60% Purina + 40% butter fat.
4	34	26	30	1.2	3.67	0.36	Killed 4 hr 45 min after feeding as above.
4	34	34	34	—	—	—	" 5 " after feeding as above.
5	27	23	25	1.1	3.56	0.35	" 7 " " " " "
4	23	22	22	1.0	3.48	0.31	" 9 " " " " "

N.F. = Neutral fat plus cholesterol.
P.L. = Total phospholipids.

TABLE III.

Effect of Feeding Non-lipid Substance on the Serum Esterase and Liver Lipids of Rats.

No. of rats	Serum esterase			Liver, %			Remarks.
	Max.	Min.	Avg	N.F.	P.L.	Chol.	
1	—	—	18	1.3	3.90	0.28	Control.
1	—	—	17	1.0	4.05	0.23	Killed 1 hr after 0.8 g of sucrose per 100 g by stomach tube.
2	21	15	18	1.7	3.78	0.24	" 2 " " feeding as above.
2	25	19	22	1.0	3.81	0.23	" 4 " " " " "
2	22	16	19	1.4	3.42	0.22	" 6 " " " " "
3	20	17	18	1.3	4.11	—	Controls.
1	—	—	15	1.4	4.40	—	Killed 1 hr after 0.5 g glycerol per 100 g by stomach tube.
4	23	12	18	1.6	4.04	—	" 2 " " feeding as above.
4	20	13	15	2.0	4.02	—	" 4 " " " " "
4	26	12	19	2.1	3.74	—	" 6 " " " " "
2	16	16	16	—	3.70	—	" 7 " 20 min. after feeding as above.
1	—	—	20	0.9	4.00	0.29	Control.
1	—	—	19	0.9	4.15	0.29	Killed 1 hr 30 min after 0.75 g proteosepeptone per 100 g by stomach tube.
2	21	18	19	1.1	4.32	0.29	Killed 2 hr 30 min after feeding as above.
2	25	25	25	1.0	4.29	0.29	" 4 " " " " " " "
2	18	15	16	1.1	4.00	0.26	" 6 " " " " " " "

N.F. = Neutral fat plus cholesterol.
P.L. = Total phospholipids.

liver have been found not to affect the esterase response. A lack of correlation between the degree of visible lipemia and the esterase value has been noticed. In many cases the lipemia would have practically disappeared while the esterase value still remained high, while in others a marked lipemia was associated with a relatively low esterase value.

In considering the results herein presented it should be realized that only changes in the tributyrin hydrolyzing power of the serum are under consideration. Mosters[3] did not find any definite correlation between the ability of blood to hydrolyze methyl butyrate and tributyrin. He found that ascorbic acid administration increased the blood's power to hydrolyze methyl butyrate but the effect on tributyrin hydrolysis was very indefinite, the two effects being sometimes in opposite directions. Gajdos,[4] however, found the subcutaneous administration of ascorbic acid to invariably increase the serum's power to hydrolyze either substrate.

Any discussion as to the possible significance of serum esterase in fat metabolism and in determining an animal's resistance or susceptibility to halogen hydrocarbons seems unjustifiable at the present time. However, further data is being accumulated in an attempt to definitely determine the significance of this enzyme in these conditions.

Summary. It has been shown that the oral administration of non-lipoid foods, glycerol, sucrose, glucose and proteose-peptone exert no demonstrable effect on the serum esterase of rats. The administration of neutral fats, oleic and palmitic acids leads to a marked increase which lasts for several hours.

The authors gratefully acknowledge their great indebtedness to the John and Mary R. Markle Foundation for financial assistance in this research.

[3] Mosters, J., *Klin. Wochschr.*, 1936, **15**, 1557.

[4] Gajdos, A., *Compt. rend. soc. biol.*, 1939, **131**, 59.

11252 P

Preparation of Antigenic Material Inducing Leucopenia from *Eberthella typhosa* Cultured in a Synthetic Medium.

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Substances presumably free from whole protein which exhibit antigenic and toxic properties have been derived from cultures of the *Salmonella* group of organisms by Boivin and his associates,[1] and Topley and his coworkers.[2] The exact chemical nature of such agents as well as several of their immunologic aspects and effects on the animal body await further investigation. The present communication describes the occurrence of certain phenomena following the injection of mice and rabbits with a toxic, antigenic material prepared from cultures of *Eberthella typhosa* grown in a synthetic medium. The use of a medium containing ingredients which could all be removed by dialysis obviously should facilitate the separation of bacterial products from constituents of more complex media of unknown composition.

Adopting as a base the inorganic salts suggested by Burrows,[3] a synthetic medium was devised of the following composition: $(NH_4)_2SO_4$, 10 g; NaCl, 1 g; K_2HPO_4, 4 g; glucose, 7 g; acid hydrolyzed casein, 12 g; tryptophane, 0.2 g; cystine, 0.4 g; phenol red (0.2% solution), 10 cc, and distilled water, 2,000 cc. The reaction was adjusted to pH 7.4 and the mixture sterilized in the autoclave, except for the glucose, which was later added as a sterile 50% solution.

A recently isolated strain of *E. typhosa* possessing the characteristics of the smooth form was employed.

Method. 14 flasks each containing 2,000 cc of the solution were inoculated with 15 cc of a 12-hour culture of *E. typhosa* that had been cultivated in this same medium for 3 successive transfers. These flasks were incubated at 37°C. At 8-hour intervals, 10 cc of a 50% solution of glucose were added and sufficient NaOH to restore the pH to approximately 7.4. After 72 hours 10 g of sodium acetate

* DeLamar Fellow.

1 Boivin, A., Mesrobeanu, I., and Mesrobeanu, L., *Compt. rend. Soc. d. Biol.*, 1933, **113**, 490.

2 Raistrick, H., and Topley, W. W. C., *Brit. J. Exp. Path.*, 1934, **15**, 113.

3 Burrows, W., *J. Inf. Dis.*, 1939, **64**, 145.

and 3 volumes of 95% alcohol were introduced into each flask which was then placed in the refrigerator for 48 hours. The supernatant fluid was decanted and the precipitate of cells and a grey, gummy material was recovered by centrifugation. These sediments from all the flasks were then combined by taking them up in 1,000 cc distilled water. The emulsion was shaken with glass beads for 2 hours at 37°C, placed in the refrigerator overnight and again shaken for 2 hours. After centrifugation, the solution was retained and the insoluble material resuspended in 300 cc of distilled water and the process repeated. The combined aqueous extracts, freed from particulate matter by centrifugation for ½ hour at 4,000 to 5,000 rpm, were placed in cellophane bags and dialyzed against running tap water at 10-14°C for 24 hours. Sodium acetate (0.5%) was added to the opalescent suspension; the reaction was made just acid to litmus with acetic acid and 3 volumes of alcohol added. After standing over night in the refrigerator, the precipitate was removed, resuspended in water and reprecipitated with 3 volumes of alcohol. This procedure was twice repeated. After the last precipitation the deposit was dissolved in 200 cc distilled water and shaken twice with 40 cc of chloroform and 8 cc of n-butyl alcohol according to the method of Sevag[4] to remove any protein present. Following the addition of 3 volumes of alcohol, the precipitate was washed twice with 70% alcohol and stirred with 100 cc of distilled water to give a light grey, colloidal suspension with a pH of 6.9 The yield was 0.8 g as determined by drying and weighing an aliquot of this solution.

Qualitative tests showed an absence of protein by the sulfosalicylic acid and HNO_3 tests. The biuret and ninhydrin tests were faintly positive. Millon and Hopkins-Cole tests were negative. A strongly positive Molisch reaction was obtained. After acid hydrolysis, treatment with phenylhydrazine yielded osazone crystals which were similar to those obtained with glucose. Elementary analyses gave: total N, 7%; amino N, 1%; total P, 5.6%. After hydrolysis with 1 N HCl for 1½ hours at 100°C, the product yielded 3.2% amino N and 13.4% reducing sugar (calculated as glucose). The ash was 24.4%.

Antigenic properties: With 3 typhoid antisera, the colloidal suspension induced a precipitate in a dilution of 1:200,000 (ring test 2 hours at 20°C). To 3 normal rabbits a total of 15 mg of this antigen in increasing doses were administered intravenously over a period of 3 weeks. Their sera exhibited agglutination titers of 1:1280 with a suspension of strain 0901 of *E. typhosa* and precipitin reactions with

[4] Sevag, M. G., *Biochem. Z.*, 1934, **273**, 419.

TABLE I.
Rabbit No. 5.

Hours after injection	Body temperature	Total WBC count
0	102.4°F	11,750
0.15 mg antigen inj. intravenously		
2	106.2	2,600
4	106.6	2,350
8	104.8	11,000
24	104.0	21,150
32	103.6	14,000

the suspension of the antigen were positive in a dilution of 1:1,000,000 of the latter.

Toxicity: 4-5 mg of the preparation injected intraäbdominally into mice weighing 15-18 g killed 90% of the animals. Five rabbits of 1.5 to 2.5 kg body weight died within a few hours after intravenous injections of 0.4-0.5 mg. Intravenous administration of 0.1-0.25 mg was followed by marked leucopenia and fever in rabbits within 1 to 2 hours, subsequently attended by leucocytosis and a drop in temperature. Table I presents the results obtained in one of 10 rabbits so treated, all of which exhibited similar reactions.

Differential counts showed that the leucopenia resulted from the almost complete disappearance of the polymorphonuclear cells from the circulating blood. During the subsequent leucocytosis, these cells rose to a high level and many of them were found to be immature as indicated by the presence of single or double lobed nuclei. A number of years ago, a leucopenia in rabbits was described by Zinsser and Tsen[5] following large intravenous doses of suspensions of typhoid bacilli. While the present work was in progress, Smith[6] and Dennis and Senekjian[7] reported the isolation of leucocidal materials from cultures of *E. typhosa.* The material studied seems to differ from the leucocidal factor recently isolated by the latter authors in that it is precipitable from an acidified aqueous solution by the addition of alcohol. Twenty-four hours following the intracutaneous injection of 1 mg of the material into normal and immune rabbits, severe inflammatory reactions 2.5 cm in diameter appeared characterized by redness and marked edema.

The toxic properties of our substance appeared to be thermostabile, since boiling for ½ hour did not alter its capacity to kill rabbits or to induce a leucopenia and fever. A study of the relationship between its toxic and immunologic characteristics is in progress.

Summary. An antigenic substance has been isolated from cul-

5 Zinsser, H., and Tsen, E., *J. Immunol.*, 1916, **2**, 247.

6 Smith, E. V., *Am. J. Hyg.*, 1939, **29** (section B), 15.

7 Dennis, E. W., and Senekjian, H., *Am. J. Hygiene*, 1939, **30** (section B), 103.

tures of *E. typhosa* grown in a synthetic medium. Appropriate amounts kill mice and rabbits and smaller quantities induce a leucopenia and fever in rabbits.

The author wishes to acknowledge with gratitude the advice of Drs. J. F. Enders, J. H. Mueller, Y. Subbarow and M. A. Logan of the Harvard University Medical School.

11253

Skin Sensitivity of Man to Bovine Plasma and Its Albumin and Globulin Fractions.

ARNOLD J. KREMEN, HENRY L. TAYLOR AND HARRY HALL. (Introduced by O. H. Wangensteen.)

From the Department of Surgery and the Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis, Minn.

The injection of whole bovine plasma in man was found to be associated with a considerable number of untoward reactions.[1] In an effort to determine the source of these reactions the whole plasma was fractionated into albumin and globulin portions. A study of skin sensitivity of man to these materials forms the basis of this paper.

Method From sterile bovine plasma, prepared as described elsewhere,[1] albumin and globulin fractions were separated by ammonium sulfate precipitation. The details of this procedure will appear in a subsequent publication.

One hundred and forty-six patients selected at random* were tested for skin sensitivity to bovine albumin solution, bovine globulin solution, and whole bovine plasma. Normal NaCl solution was used as a control. Five-hundredths cc of each of the above was injected intradermally and readings were taken after 20 to 30 minutes. No change or disappearance of the original wheal was considered negative, slight enlargement of the wheal with a small area of erythema 1+, a marked halo of erythema without pseudopodia 2+, pseudopodia and enlargement of the original wheal to twice original size

[1] Wangensteen, O. H., Hall, Harry, Kremen, A. J., and Stevens, B., in press.

* Acknowledgment is made to Dr. Macnider Wetherby, Head of the Outpatient Department of the University of Minnesota Hospitals, for his cooperation in this study.

TABLE I.
Results of Skin Tests.

Solution used	Reactions 0 No.	0 %	1+ No.	1+ %	2+ No.	2+ %	3+ No.	3+ %	4+ No.	4+ %
Saline solution	136	93	7	5	3	2				
Bovine albumin solution	99	68	39	27	8	5				
" globulin "	56	38	50	35	36	25	3	2	1	0.7
Whole bovine plasma	25	17	43	30	53	36	23	15.5	2	1.5

TABLE II.
Relationship of Type of Reaction with Albumin and Globulin Fractions to Type of Reaction with Whole Plasma.

Plasma Reaction	Type of albumin reaction Figures expressed in % 0	1+	2+	Type of globulin reaction Figures expressed in % 0	1+	2+	4+
0	100			100			
1+	100			63	37		
2+	42	49	9	35	50	15	
3+	35	50	15	20	75	5	
4+		50	50		50		50

3+, and marked edema, induration, pseudopodia and erythema 4+.

Results. The results of skin tests on 146 patients are tabulated in Table I. It is noted that a wide difference of skin sensitivity appears between the albumin, globulin and whole plasma. There were 7% positive reactions (1+ to 2+) with the control saline solution. The albumin caused the least number of positive reactions, the globulin was intermediate, and the whole plasma caused the largest number of positive reactions as well as those of greatest magnitude.

Table II shows the relationship of the reactions of the albumin and globulin fractions to those of whole plasma. The results are tabulated as the percentage of the type of reaction of the plasma fractions that occurred with the different types of reaction to whole plasma. In no instance did the type of reaction of the albumin or globulin fraction exceed that of the whole plasma in intensity. In only 4 cases (2.7%) was the reaction of the albumin equal to that of the plasma and in 34 cases (23.3%) was the reaction of the globulin fraction equal to that of the whole plasma.

Summary and Conclusions. 1. The skin sensitivity of man to bovine plasma and to its albumin and globulin fractions has been investigated. 2. The skin sensitivity of man to the three preparations tested is least marked with the albumin fraction of bovine plasma.

11254 P

Evacuation of the Gall Bladder in Patients with Pernicious Anemia.*

J. A. Layne and E. A. Boyden.

From the Departments of Medicine and Anatomy, University of Minnesota, Minneapolis.

This is the third of a series of studies analyzing the rate of emptying of the gall bladder in patients with lesions of the stomach.

In the first series[1] it was observed that peptic ulcer patients displayed a significantly faster rate of emptying than controls of comparable age. Since the mean curve of emptying for this group could be virtually reproduced in normal individuals merely by injecting one egg-yolk directly into the duodenum (Fig. 1, left) and since the initial discharge of food into the duodenum is known to be more rapid in individuals having gastric and duodenal ulcer, the faster rate of emptying of the gall bladder in such patients was attributed to a greater food stimulus rather than to an increased production of gastric juice.

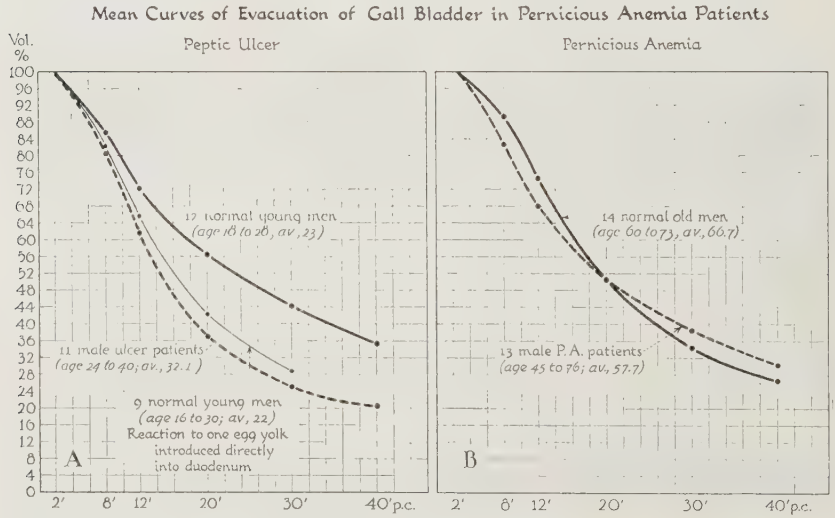

Fig. 1.

Mean curves of the evacuation of the gall bladder in patients with lesions of the stomach. Ordinates, percentage volume of bile in the gall bladder; abscissæ, minutes after a standard meal.

* Aided by grants from the Medical Research Fund of the Graduate School.

1 Boyden, E. A., and Berman, T. M., *Radiol.*, 1937, **28**, 273.

This interpretation was strengthened by the second study.[2] This dealt with patients having carcinoma of the stomach. In this group, marked reduction of the amount of free HCl failed to retard the emptying of the gall bladder.

The present report is based upon cholecystographic studies of 22 consecutive, unselected male patients for whom a clinical as well as hematological diagnosis of pernicious anemia had been established. All of the patients were receiving treatment with liver extract. The hemoglobin content of the blood had either reached normal levels or was responding to treatment. The histamine test had disclosed complete absence of free acid in all patients, and only minimal amounts of total acids.

Of special interest was the fact that the gall bladder could not be visualized in 40% of these individuals, notwithstanding the use of the intravenous method of introducing the dye and the absence of any history suggesting gall bladder disease. In the other groups visualization had failed in only 9% of ulcer patients and in 22.7% of those with carcinoma.

In the remaining 13 of the 22 patients, the mean curve of emptying of the gall bladder approximated that of the controls (Fig. 1, right), being a little faster in the first 20 minutes after a standard meal† and a little slower in the last 20 minutes, but not significantly so.

Since there was complete absence of free HCl in all 22 patients, even after stimulation by histamine, one must conclude that free HCl in the stomach is not an essential factor in the evacuation of the human gall bladder.

The final report will include a comparable study of female patients.

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2 Ritchie, W. P., and Boyden, E. A., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 815.

† The modified Boyden meal, consisting of 4 egg-yolks mixed with an equal amount of milk to which a pinch of sugar and a drop of vanilla extract has been added.

11255 P

Poliomyelitis in *Sigmodon hispidus littoralis* Rats.*

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Recently, Armstrong[1] reported that he produced poliomyelitis in *Sigmodon hispidus hispidus* rats (Eastern cotton rats) by injecting poliomyelitis virus (Lansing strain). He was unsuccessful when other strains were used. It is almost impossible to distinguish this animal from *Sigmodon hispidus littoralis,* an animal more common in the South and relatively inexpensive.

Ten rats *(Sigmodon hispidus littoralis)* were given a 10% suspension of Flexner's M.V. strain, 5 getting purified and eluated virus and 5 unpurified virus. Armstrong's technic was followed, the doses in each instance in the following experiments being 0.06 cc intracerebrally, 0.06 cc intranasally and 0.5 cc subcutaneously. Two of the animals which received unpurified virus died 5 days later. Pathologically, the central nervous system showed nothing that resembled poliomyelitis. A suspension of the brain and cord obtained from these animals was injected into 5 rats. These remained normal after nearly 3 months of observation.

Nine virus strains [Flexner's M.V., Toomey, Flexner (Phil., Wolman), Kramer, Harmon, Howitt, Trask W.E., Flexner W.E., Australian] were each injected into 5 rats. One rat given Flexner M.V. virus died the second day after injection; 1 given Flexner (Phil.) virus died the sixteenth day; 1 Kramer, the sixth day; 1 Harmon, the second day; 1 Harmon, the ninth day; 1 Howitt, the fifth day; 1 Flexner W.E., the fourth day; 1 Australian, the fifth day. There was nothing found in any of the cord sections from these animals that resembled experimental poliomyelitis. The animals that died during the first few days after injection had some local intracerebral hemorrhage. Their deaths were probably due to trauma.

After these 8 animals died the cord and brain of each were emulsified and injected into 3 rats (24 in all). After 2 months of observation the animals were still alive.

Nearly 3 months after the original experiment with the Flexner M.V. virus strain or 2 months after injecting the other 9 poliomyelitis strains, 59 of the 74 surviving rats were injected with Arm-

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

1 Armstrong, Charles, *Pub. Health Rep.,* 1939, **54**, 1719.

strong's Lansing virus strain. Concomitantly, 10 rats never previously injected were used as controls. The animals began to die within 4 days after the injection. Every animal but one of the 69 succumbed within 10 days. The rats showed all types of paralyses, the most common one being bulbar. There were some which had incoördination and peculiar rolling or circling movements as well. The pathologic findings were the same as those described by Lillie and Armstrong.[2] However, there was not much perivascular infiltration.

Armstrong's virus (Lansing) was carried through 5 generations of rats in only 22 days, transmission being made every fourth day.

Conclusion. The Lansing strain of virus isolated by Armstrong easily produces disease in *Sigmodon hispidus littoralis* rats. Thus far, we have been unable to produce poliomyelitis with any of the 9 stock virus strains carried in our laboratory and named in this paper.

11256 P

Quantitative Alteration in Renal Corpuscles of Salamanders Through Variation in Hypophyseal Tissue.*

RAYMOND F. BLOUNT.

From the Department of Anatomy, University of Minnesota, Minneapolis.

Previous reports have shown that increasing the amount of hypophyseal tissue in an amphibian embryo by grafting gives rise to an increase in the thickness of the basement membrane of the renal glomerulus.[1] It has been pointed out that this thickening is similar in appearance to the thickened membrane in human cases of hypertension.[1, 2] The size of the heart, vasoconstriction, reduced heart rate and other conditions in these test animals simulate a hypertensive state.[3] The hypophysectomized individuals show the opposite results of hypotension and may now be stated to have correlated with this a thin basement membrane.

2 Lillie, R. D., and Armstrong, Charles, *Pub. Health Rep.*, 1940, **55**, 115.

* Aided by a grant from the research funds of the Graduate School, University of Minnesota.

1 Blount, Raymond F., *Anat. Rec.*, 1936, **65**, 1.

2 McGregor, Leone, *Am. J. Path.*, 1930, **6**, 347.

3 Blount, Raymond F., *J. Exp. Zool.*, 1935, **71**, 421.

The arrangement of the loops of the capillary tuft indicates that there is a simplification in these hypertensive glomeruli.[4] This is stated by McGregor[2] to be true in human hypertension. She also states that there is contraction of glomeruli although no measurements or volume determinations were made. Goormaghtigh[5] mentions contraction of glomeruli in ischemic kidneys.

In our test animals there appeared to be a contraction in many glomeruli with an increase in the capsular space. How much this increase in space is due to a shrinkage of the glomerulus and how much to an increase in capsular volume had to be determined. The present observations deal with quantitative determinations of volume for the total renal corpuscle, the glomerulus and the capsular space of hypophysectomized, control, and triple pituitary animals. This has been done by the paper cut-out method using every serial section. Two groups of animals were employed. In one, values for every fifth corpuscle were computed individually in 3 sets each consisting of an hypophysectomized, a control and a triple pituitary animal. In all there were 153 corpuscles for which computations were made. In the other series the volumes for all the corpuscles were determined and the averages obtained for the 12 animals of 4 sets.

The general magnitude of one of these glomeruli is about .000150 cu mm. However, considerable variation was found in the volume since there were extremes of .000011 and .000880 cu mm. The larger glomeruli were at the cranial pole of the kidney and the smaller ones at the caudal. Although the smallest glomeruli were found in the triple pituitary animals some few were large. The cranio-caudal gradation in glomerular size was not a factor in the differences in the test animals since the distribution of glomeruli, as to myotome level, was about the same in all 3 types. There was no definite relationship between body size and glomerular size. However, the amount of body volume per glomerulus was greater in the larger animals.

The percentage relationship that the average glomerulus bears to the entire corpuscle is less in the triple pituitary than in the control animal in all the cases except one which is mediocre by other criteria. This animal follows the trend if the mode rather than averages is used. In the hypophysectomized individuals the glomerulus occupies a greater proportion of the capsule in all but 2 cases, the above set and one other. Some glomeruli of these animals show enlargement due to vasodilation. The percentages, in the 4 sets on which values

[4] Blount, Raymond F., *Anat. Rec.*, 1937, **67**, Suppl. No. 3, 7.

[5] Goormaghtigh, N., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 688.

were obtained for all glomeruli, are in the hypophysectomized, control and triple pituitary animals respectively as follows: 79, 74, 49; 81, 65, 39; 75, 68, 61; and 76, 67, 51. In 2 of these 4 sets the relatively larger capsular space, in the triple pituitary animal, results from the glomerulus' being contracted and in the other 2 from the great increase in the volume of the capsule. Stasis is probably responsible for the large size of some of the triple pituitary glomeruli. In all cases the generally thickened membrane (up to 8 times normal) balances to some extent the contraction of the capillary tuft in determining the glomerular volume.

These observations show that the renal corpuscle of animals with an increased amount of pituitary tissue have glomeruli that often but not always exhibit contraction, and that there is a clear-cut reduction in the relative size of these glomeruli. In the hypophysectomized individuals the corpuscles show no uniform alteration in glomerular size but a definite tendency toward a reduction in the relative size of the capsular space.

11257 P

Parasympathomimetic Effects of Erythrocytes in the Cat.

M. B. BENDER.* (Introduced by Gregory Shwartzman.)

From the Laboratories of the Mount Sinai Hospital, New York.

Using the completely denervated iris of the cat as an indicator of circulating autonomic substances, it was found that constriction of the pupil ensued from 35 to 70 seconds following the intravenous administration of 1.0 cc of whole human blood. The blood of rabbit, guinea pig, mouse, monkey, hog and hemolyzed erythrocytes of cat also produced a constriction of the completely denervated iris of the cat. Identical amounts of blood from dog, sheep and ox were without significant effect on the same preparation. The most conspicuous reactions were obtained with rabbit's blood. Large quantities of dog, ox, or sheep blood sometimes produced a slight and slow constriction of the cat's denervated iris.

Other effects caused by the intravenous injection of blood into the cat were a fall in blood pressure, as shown by Abramson, *et al.*,[1] and

* This investigation has been aided by grants of the Josiah Macy, Jr., Foundation and the Dazian Foundation for Medical Research.

1 Abramson, D. I., Wasserman, P., and Senior, F. A., *Am. J. Physiol.*, 1938, **124**, 402.

Fuzii,[2] decrease in heart rate, relaxation of anal and urinary sphincters and hypersalivation, all parasympathomimetic manifestations.

The vaso depressor and miotic properties of the blood were located chiefly in the erythrocytes. Laked red blood cells also exhibited these properties. Intravenous injection of serum never produced a visible change in the iris.

The washed red blood cells caused a pupillary constriction from 35 to 80 seconds after their intravenous injection, whereas the laked erythrocytes produced a much quicker response, the miosis appearing from 12 to 30 seconds after the administration. The rabbit's erythrocytes showed two types of depressor effects on the cat's blood pressure, an early transient drop from 6 to 10 seconds after the injection of the cells and a later prolonged fall from 45 to 70 seconds after the administration. The hemolyzed red blood cells showed only the early depressor effect. The findings concerning the effect upon blood pressure were similar to those of Fuzii.[2]

Furthermore, the cat's own laked blood produced an early and transient drop in its blood pressure, and a constriction of its completely denervated iris. The smallest quantity of rabbit's blood necessary to produce a constriction of the denervated iris was 0.5 cc of one percent saline suspension of erythrocytes per kilo of body weight of cat. The intact cells were more effective in producing parasympathomimetic reactions than the laked erythrocytes. By centrifuging the hemolyzed red blood cells of the rabbit, it was found that the constrictor factor of the blood was also present in the supernatant red fluid.

Since the iris was completely denervated, the miotic action of the injected bloods mentioned must be due to a chemical agent. The substance had no effect on denervated facial striated muscle or on the radial smooth muscle of the iris denervated by the removal of the superior cervical ganglion. The constriction of the pupil by the intravenous injection of erythrocytes was demonstrated in the eye in which either the oculomotor nerves (preganglionic) or ciliary nerves (post-ganglionic) were acutely sectioned. Atropine sulfate did not hinder the miosis produced by larger quantities of blood. Eserine did not seem to augment the reaction.

Intravenous injections of whole blood, erythrocytes, or laked corpuscles of the species mentioned produced no reaction in the completely denervated iris of the monkey.

I am indebted to Dr. Gregory Shwartzman for his valuable aid and suggestions in this problem.

[2] Fuzii, K., *Tohoku J. Exp. Med.*, 1939, **35**, 384.

11258 P

Detection of Human Influenza Virus in Throat Washings by Immunity Response in Syrian Hamster *(Cricetus auratus)*.

R. M. Taylor. (Introduced by J. H. Bauer.)

From the Laboratories of the International Health Division of the Rockefeller Foundation, New York.

In a previous communication[1] the serial passage of the human influenzal virus in the European hamster *(Cricetus cricetus)* was reported. While this hamster was shown to be susceptible to a recently isolated virus strain, it was not demonstrated, because of lack of material at the time, whether or not infection could be transferred directly from humans.

This present report deals with the infection of the Syrian hamster *(Cricetus auratus)* from throat-washings of persons ill with epidemic influenza.

Material. The washings were collected in buffered broth during the early months of 1939 from persons acutely ill with influenza. The presence of virus originally in these specimens had been demonstrated by the inoculation of ferrets.[2] The washings had been preserved for 9 to 10 months in a low-temperature cabinet at –76°C when the inoculation of the hamsters was made. The hamsters employed were from 3 to 5 months of age and weighed approximately 100 g each.

Method. Preceding inoculation, 1 cc of blood was withdrawn by cardiac puncture from each hamster. From 0.3 to 0.4 cc of unfiltered throat-washing was administered intranasally while the animal was under light ether-anesthesia. Twelve to 14 days after the intranasal inoculation a second blood sample was taken. The blood serum obtained before and following inoculation was then titrated for neutralizing antibodies against the PR8 strain of influenzal virus.

Results. So far, 4 preserved washings obtained during the 1939 epidemic, but from separate localities, have been tested. All of these specimens were known to contain virus originally.

None of the blood samples taken before inoculation showed any neutralizing effect upon the virus in a 1 :2 serum-mixture, but samples taken 12 to 14 days after inoculation of the hamsters with each of these 4 washings all neutralized 1000 M.L.D. of the virus in dilu-

1 Taylor, R. M., and Dreguss, M., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 100.

2 Horsfall, F. L., Jr., Hahn, R. G., and Rickard, E. R., *J. Clin. Inv.*, 1940, **19**, 379.

tions above 1 :32. Despite the immune response, these animals manifested no symptoms or gross pathological lesions which could be used as diagnostic criteria.

Three washings which did not contain influenzal virus as shown by ferret-inoculation failed to stimulate neutralizing antibodies in the hamster. There appears to be little doubt, therefore, that the immune response in the hamster was specific and resulted from influenza-virus infection. Moreover, the infection established through the inoculation of throat-washings may be transferred from one hamster to another.

Hitherto the ferret has been used almost exclusively for the detection of the virus in human material, either by immunity response or by subsequent passage and identification in mice. The virus has been obtained directly in white mice from human throat-washings,[3] but this is a tedious and unreliable method. Several mouse-passages are required before the virus becomes manifest, and, in the experience of the author, only a small proportion of strains which may be identified in throat-washings by means of the ferret can be obtained by direct inoculation of mice. Nor has it been possible in this laboratory to demonstrate the development of immunity in mice inoculated with washings known to contain the virus.

Thus the hamster is the only animal other than the ferret in which it has been shown that the presence of virus in human throat-washings may be detected by the immunity response to the original inoculum. The relatively low cost of the hamster, the ease with which it may be bred in the laboratory, and its apparent resistance to canine distemper and other epizoötics to which the ferret is so subject would make it a welcome substitute for the ferret. However, it is realized that these observations must be extended before it can be determined to what degree the hamster may be used as an adjunct or substitute for the ferret in the study of human influenza.

It may be added that 3 of the throat-washings which produced an immunity-response in the hamsters were at the same time administered intranasally to cotton rats *(Sigmodon hispidus hispidus)*. Two of the rats showed no rise in antibodies, and the third gave only a minimal and questionable response.

[3] Francis, T., Jr., and Magill, T. P., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 132.

11259 P

Growth and Differentiation of Daphnia Magna Eggs *in vitro*.

Vasil Obreshkove and Alan W. Fraser.
From Bard College, Columbia University.

In the course of some ultra-centrifuging experiments there arose the need of rearing the parthenogenetic eggs of Cladocera *in vitro*. A review of the literature revealed no record of the successful growth of eggs of this group of animals outside the brood-chamber of the mother. We have succeeded in rearing the eggs of *Daphnia magna* in tissue culture slides and inasmuch as the embryos are transparent, this enabled us to make observations on some of the sequence of events in the course of the development of the embryos without the necessity of staining and sectioning.

A number of different media were used in an endeavor to grow *Daphnia magna* eggs *in vitro*. A modified Ringer's solution utilized successfully for physiological work on isolated organs of Cladocera by Levy,[1] proved injurious to the eggs. The growth of eggs in a medium consisting of the fluid obtained from the brood-chamber of animals in which there were developing eggs showed no advantage over a simpler medium consisting of sterile pond water. This is of considerable interest in view of the fact that it has been held (Dearborn,[2] Birge[3]) that the brood-chamber of Cladocera secretes a fluid which serves as nourishment in the course of the development of the embryos. Careful controlled experiments revealed that the parthenogenetic eggs in the brood-chamber of *Daphnia magna* completed their development in about 46 hours at 25°C. When the parthenogenetic eggs of this animal were reared in sterile pond water on tissue culture slides, they too completed their embryonic history from eggs to free swimming independent organisms in the same period of time. This demonstrates very clearly that the eggs of this animal are self-sufficient with regard to the nutritive material already stored in them at the time of their deposition.

A *Daphnia magna* female, the eggs of which were to be removed from the brood-chamber, was placed in a depression slide with round polished cavity 15 mm in diameter and 3 mm deep filled with sterile

1 Levy, R., *Compt. Rend. Soc. Biol.*, 1927, **97**, 1600.

2 Dearborn, G. V. N., Chapter on protoplasm and simple animal functions. *Human Physiology*, Philadelphia and New York, Lea & Febiger, 1909.

3 Birge, E. A., Chapter on water fleas, *Fresh Water Biology*, Ward and Whipple, New York, John Wiley & Son, 1918.

pond water. With the aid of 2 fine needles the animal was held in place in the field of the binocular microscope and the ventral edges of the carapace were carefully spread apart. By gently moving the body of the animal back and forth with the needles, the eggs were made to roll out of the chamber without being subjected to any pressure. The eggs were transferred to a new depression slide containing sterile pond water and were incubated at 25°C. The chorion and the vitelline membranes of the eggs are very easily injured. The removal of eggs from the brood-chamber of the mother without injury to them determines the degree of success of rearing the eggs of this animal *in vitro*.

We have obtained several complete series of photomicrographs of *Daphnia magna* eggs taken at 3-hour intervals throughout the course of their development *in vitro*. We have made observations on some of the more prominent changes in the course of the development of the living material as observed by gross microscopical examination. The first sign of movement of the body was observed in embryos 30 hours old. At this stage also the heart exhibited its first pulsations. The first external evidence of brain development appears as a blastodermic thickening mid-dorsally in the cephalic region of 21-hour-old embryos. In embryos 27 hours old there comes into prominence, dorsal to the prospective brain, a double mass of granular substance, representing the material for the development of the eye. In adult individuals the eye is a single organ placed in front of the head but embryologically this structure has a double origin. Other details in the course of the development of the embryos *in vitro* were also followed. Embryos reared on depression slides when transferred to bottles containing the standard amount of the culture medium (Banta[4]) become sexually mature and produce young.

[4] Banta, A. M., *Physiology, Genetics and Evolution of Cladocera*, Carnegie Institution Publication No. 513, 1939.

11260

Changes in Structural Components of Human Body from Six Lunar Months to Maturity

HARRY A. WILMER. (Introduced by R. E. Scammon.)

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The changes of the various structural components of the human body in development are well illustrated by volumetric histograms. These are shown in Figs. 1 and 2. They are based upon data collected in this laboratory as well as a series of records collated from the literature.

In this presentation the structural components of the body considered are: skin and superficial fat *(paniculus adiposus)*, the visceral mass (including the heart), the nervous tissues (of both the peripheral and the central nervous systems), the voluntary musculature, and the fresh ligamentous skeleton.

The periods represented are: 6 lunar or fetal months, birth, and full maturity. Both sexes are included in each of the periods.

Figure 1 illustrates the differences in composition of the body at 6 lunar months and at birth. During this interval the net weight of the body increases approximately five-fold, from 500 to 2500 g.

The relative changes in the distribution of the body components are: the skin and superficial fat increase from about one-sixth to over one-fourth of the body mass. The nervous tissue component and the

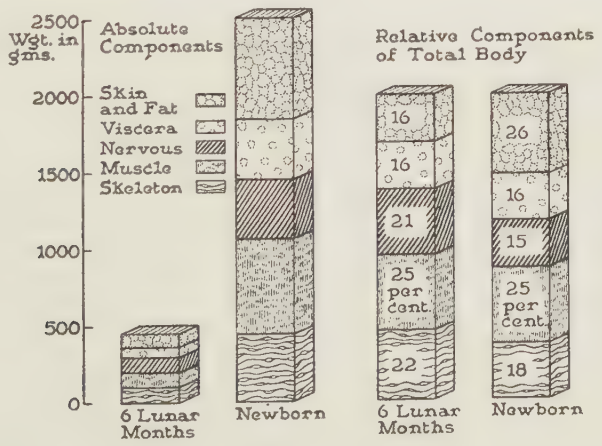

FIG. 1.

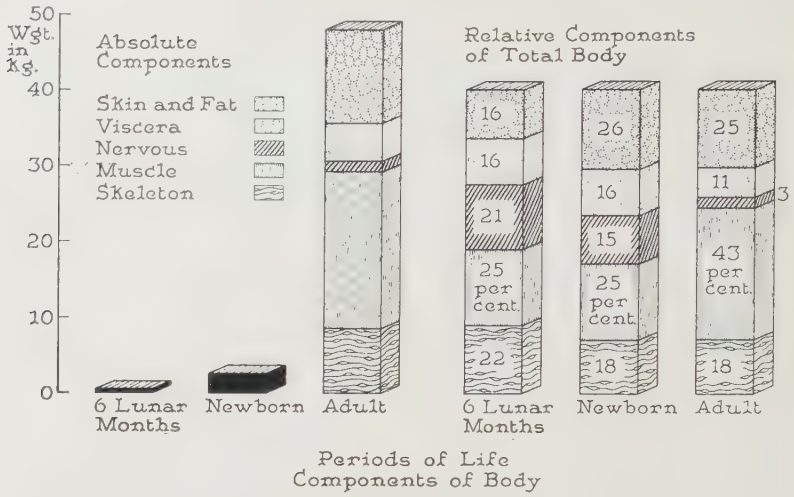

FIG. 2.

skeletal component both show a marked relative decrease. The visceral and the voluntary muscle components remain almost constant.

Fig. 2 shows the changes in components between birth and maturity. During this interval the net body weight increases almost twenty-fold. The postnatal changes in the relative distribution of components consist of: a marked increase in the voluntary muscle from about one-fourth to over two-fifths of the body mass. But the visceral component decreases from about one-sixth to about one-tenth of the body mass; and the nervous tissue component from about one-seventh to about one-thirtieth. The skin and superficial fat component and the component of the skeleton remain practically unchanged.

TABLE I.

Absolute and Relative Weights of the Various Components of the Body for Both Sexes. (Collated from Various Sources.)

Body components	Absolute wt (g)			Relative wt (%)		
	6 lunar months	Newborn	Adult	6 lunar months	Newborn	Adult
Skin and superficial fat	73.5	666.35	12,395.5	16.21	26.43	25.61
Muscle	111.5	625.25	20,846.3	24.60	24.80	43.07
Skeleton	101.0	446.00	8,518.6	22.28	17.69	17.60
Total viscera	71.1	398.20	5,185.5	15.68	15.79	10.71
Central nervous system	96.2	385.50	1,455.9	21.22	15.29	3.01
Sum*	453.3	2,521.30	48,401.8	100.00	100.00	100.00

* These sums do not include the weight of the blood or the weight lost in the determination of approximately 33 g in the 6 months fetus, 116 g in the newborn, 5270 g in the adult male and 4000 g in the adult female.

The numerical values for the absolute and relative sizes of all these components are shown in Table I.

The most striking of this series of changes is the increase of the range of variability of the several components forming the body. At 6 lunar months there is a maximum difference of only 9% in their relative distribution. By birth there is a maximum difference of 40%. And at maturity there is a maximum difference of over 1000%. These computations are in terms of the smallest component in each series, but they may also be demonstrated in terms of the largest component or in terms of absolute values.

11261

Keto-Reacting Substances in the Bile of Dogs.

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A study of the literature revealed that certain investigators have identified and isolated various ketonic acids from the bile of different animals. Fernholz[1] isolated 3-hydroxy-6-ketocholanic acid from hog bile and studied its properties. Schoenheimer and others[2, 3] confirmed the isolation of this ketonic acid, and further stated that the ketonic acids of hog bile comprise approximately 10% of the total crude acids. Wieland and Kishi[4] isolated and identified 3-hydroxy-12-ketocholanic acid from ox bile. Sobotka found 3-hydroxy-12-ketocholanic acid in human bile.[5]

No one apparently has reported a method for the quantitative determination of carbonyl or keto-reacting substances in bile. Since our work required such a method, we have been fortunate at having at our disposal a quantitative method which had been developed by Dr. Gustus of the Wilson Laboratories, Chicago. The principle of this method is as follows: The carbonyl or keto groups in the bile are allowed to react with an excess of hydroxylamine. The remaining hydroxylamine is combined with diacetyl-monoxime converting the

1 Fernholz, E., *H.-S. Z. f. Physiol. Chem.*, 1935, **232**, 202.

2 Schoenheimer, R., and Johnston, C. G., *J. Biol. Chem.*, 1937, **120**, 499.

3 Anchel, M., and Schoenheimer, R., *J. Biol. Chem.*, 1938, **124**, 609.

4 Wieland, H., and Kishi, S., *H.-S. Z. f. Physiol. Chem.*, 1933, **214**, 47.

5 Sobotka, H., *Chem. Rev.*, 1934, **15**, 334.

latter to the dioxime compound. The dioxime is precipitated with nickel acetate, and the resulting precipitate washed, dried and weighed. By calculation, the amount of carbonyl groups that was originally present in the bile and that combined with the hydroxylamine can be obtained. The exact details of this analysis for carbonyl groups in bile will be published later by Dr. Gustus.

The accuracy of the method has been examined in two ways. First, the chemist was supplied with duplicate unknowns of the same bile obtained from 11 bile fistula dogs (Rous-McMaster method), some of which received no bile salts, and others, bile salts containing varying amounts of ketocholanic acid. The analyses of the duplicate unknowns checked within $\pm 6\%$ of the mean, except in one case in which the error was $\pm 15\%$. Second, pure triketocholanic acid added to dogs' bile was recovered in amounts varying from 95% to 99%.

Control output of keto-reacting substances. Before ascertaining the effect of feeding natural and oxidized bile acids on the keto-reacting substances in bile, it was necessary to determine the amount of keto-reacting substances present in "control" bile. By "control" bile is meant the bile that is secreted when the animal is fed the standard diet only and no bile or bile salts are administered. This was done in 55 tests on 15 dogs. The average control daily output of keto-reacting substances (Table I) amounted to 252 ± 16 mg (S.E.M.) expressed as triketocholanic acid, or 52.6 ± 4.4 mg of carbonyl groups ($252 \times .209 = 52.6$). The daily keto output was subject to considerable individual variation under basal conditions, so that the output of keto-reacting substances ranged from 98 to 507 mg per 24 hours in the different dogs. One of the dogs showed a 50% variation between different control periods; the others varied only from 5 to 15%.

Output of keto-reacting substances when natural ox-bile salts were administered. When natural ox-bile salts were administered, either in 3 or 5 g daily doses, there was an increase in keto groups secreted in the bile, *i. e.*, 72.1 mg and 92.8 mg respectively as compared to 52.6 mg for "control" bile (Table I). The source of the increased keto-reacting substances in bile is problematic. However, as has been shown before and confirmed in this work, when unoxidized cholates are given about 10% are "lost" during one enterohepatic circuit. This loss of 10% is approximately balanced by the increase of 20 mg of keto groups (Table I). This is true only when it is assumed that the natural conjugated bile acids "lost" are changed to oxidized conjugated bile acids which contain 11.2% keto groups. This is reasonable because if the natural conjugated bile acids in cattle bile are oxidized they contain 11.2% keto groups.

TABLE I.

Effect of Unoxidized and Oxidized Bile Acids on Content of Keto-reacting Substances in Dog's Bile.

Procedure	No. of dogs	No. of tests	Bile, cc/24 hr	Total C = O groups, mg/24 hr	Increase C = O groups over control	Recov. of keto acid fed	% recov.
Control: Diet without bile salts	15	55	126	52.6			
1.5 g cholic acid as cattle bile*	9	34	190	72.1	19.5		
3.0 g oxidized conjugated bile salts as they occur in cattle bile. 11.2% C = O	11	14	175	107.2	54.6†	488‡	16§
3.0 g ketocholanic acid from cattle bile. 18.3% C = O	7	10	251	186.2	133.6	730	24
3.0 g dehydrocholic acid. 20.9% C = O	6	10	264	227.3	174.7	836	28

*Contains no keto-reacting substances.

†107.2 — 52.6 = 54.6.

‡ 54.6 ÷ 11.2 × 100 = 488.
133.6 ÷ 18.3 × 100 = 730.

§488 ÷ 3000 = 16%. Note that the per cent recovered is about proportional to the keto acid content of the oxidized bile acids fed.

Output of keto-reacting substances when oxidized or ketocholanic acids were administered. We used a product containing oxidized conjugated bile acids made from cattle bile (Dechacid No. 14, Wilson Laboratories, Chicago). That is, cattle bile was oxidized without splitting-off the taurine and glycine. This product contained by analysis 11.2% keto or carbonyl groups. We used another product containing oxidized unconjugated bile acids made from cattle bile (Ketochol, Searle, Chicago). This product contained by analysis 18.3% keto or carbonyl groups. We also used pure dehydrocholic acid (Decholin, Riedel-de Haen, New York), which contained by analysis 20.9% carbonyl groups.

When the oxidized bile acids were fed a marked increase in the keto-reacting substances in the bile of the dogs resulted (Table I). By subtracting the control output of keto-reacting substances from the total output, one may obtain the increase due to the keto-acids fed. This increase may be assumed to be the amount of keto-acid fed that is recovered in the bile. In this way the recovery of the keto acid fed may be estimated. Though two other assumptions are possible, we believe the one mentioned is the most logical, since the increase in keto-reacting substances in bile after unoxidized bile acids are fed appears to be due to oxidation of the bile acid lost during an enterohepatic circuit rather than to choleresis *per se*.

Conclusions. (1) Keto-reacting substances are present in dogs' bile and can be quantitatively determined. (2) The output of keto-reacting substances is increased slightly when unoxidized bile acids are fed. (3) When oxidized bile acids or ketocholanic acids are administered orally, the output of keto-reacting substances in the bile is markedly increased. By assuming that the increase over the control output is due to the ketocholanic acid fed, one may calculate the recovery in the bile of the ketocholanic acid administered.

11262

Production of Neutropenia in Swiss Mice by Injection of Potassium Dicarboxy-benzanthracene.

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Leukopenia, found in one of Dr. A. T. Bradbury's Swiss mice dying after the injection of dicarboxy-benzanthracene, led to the investigation of the blood changes produced by this drug.

Swiss mice, both male and female, were used, the 9 controls being kept in the same cages and on the same food as the injected animals. Blood was taken from the tail, using the standard technic, and United States Bureau of Standards checked apparatus. The 9 experimental animals received subcutaneous injections of 5 mg of water-soluble potassium salt of dicarboxy-benzanthracene on 2 occasions, one week apart. The mice and corresponding controls varied in age from 1½ to 3½ months.

In normal Swiss mice, untreated, the average of 36 red blood cell counts on 18 mice was 8,930,000 ± 1,150,000 per cu mm. The average of 86 white blood cell counts on 18 normal Swiss mice was 10,100 ± 3,600 per cu mm. The absolute number of polymorphonuclear neutrophils (average of 94 counts on 18 untreated animals) was 5,848 ± 2,713 per cu mm.

One week after a single injection of the soluble potassium salt of dicarboxy-benzanthracene the average total white blood cell count fell to 8,655 ± 4,000 per cu mm, and in 2 weeks the average fell to 7,300 ± 2,600 per cu mm. The decrease was primarily the result of a fall in the number of neutrophils, as shown in Table I.

The eosinophils averaged 153.2 per cu mm in 93 counts in non-treated mice, with extremes of 0 to 510 per cu mm. The variation after injection of the benzanthracene solution was not significant.

TABLE I.

Control period— Absolute No. of neutrophils	5,448 ± 2,713 per mm³
One week after first injection	4,569 ± 2,100 " "
Second week after first injection	2,623 ± 1,070
Received second injection at this time.	
Third week after first injection	1,299 ± 627
Fifth " " " "	1,650 ± 890
Sixth " " " "	3,613 ± 1,585
Seventh " " " "	4,437 ± 2,215
Eighth " " " "	5,518 ± 957

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Before treatment, the average number of monocytes was 702.4 per cu mm (average 6.8%; extremes 1-11%). The variations 1, 2, 3, 5, 6, 7, and 8 weeks after the first injection were (averages) 672, 588, 510, 417, 678, 652, and 903 per cu mm.

Before treatment the lymphocytes averaged 3,735 per cu mm (36.16%; extremes, 15 and 62%). The variations 1, 2, 3, 5, 6, 7, and 8 weeks after the first injection were (averages) 3241, 4281, 4283, 4201, 4953, 3829, 3189 per cu mm. Thus, while the lymphocytes were as high as 70 to 80% during the post-injection period, the absolute numbers did not vary greatly, the maximum increase (average) being 1218 per cu mm.

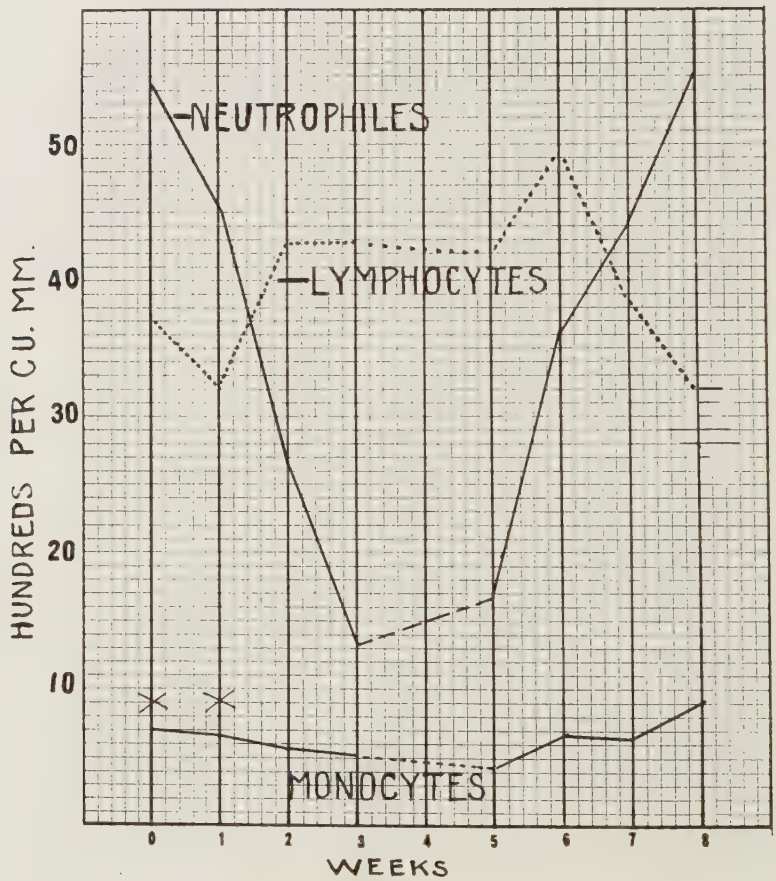

FIG. 1.

The leukocyte changes after injection of dicarboxy-benzanthracene. The curves represent the averages for 9 mice.

The point marked "O" represents the average for a control period of 2 weeks. Injections were made at the points marked "X."

There was no significant change in the number of basophils, which varied from 0 to 1%.

Summary and Conclusions. After the injection of the water-soluble potassium salt of dicarboxy-benzanthracene into Swiss mice there is a marked decrease in the number of polymorphonuclear neutrophils, a slower, and moderate decrease in the number of monocytes, with but slight changes in the number of lymphocytes, eosinophils, and basophils. Recovery is complete 7 weeks after the last injection. Data on the blood of normal Swiss mice are given.

11263

Relation Between Growth of Pneumococcus III and Concentration of Capsular Polysaccharide Appearing in Culture Filtrates.*‡

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A method of adapting the photronreflectometer[1] to the quantitative determination of pneumococcal capsular polysaccharide in solutions of unknown concentration has been described[2] In the present investigation, the method was employed to determine the concentrations of SSS III appearing in Seitz filtrates of blood broth cultures, following inoculation with varying amounts of a standardized strain of Pneumococcus III; these concentrations were correlated with the phase and amount of growth of the organisms.

A strain of Pneumococcus III obtained from the sputum of a pneumonia patient was brought to constant virulence after the suggestions of Schmidt and Hilles.[3] 5×10^{-4} ml of a 12-hour blood-

* These studies received financial support from the Metropolitan Life Insurance Company, and from Mr. Bernard M. Baruch, Mr. Bernard M. Baruch, Jr., Miss Belle N. Baruch, and Mrs. H. Robert Samstag.

† Littauer Fellow in Pneumonia Research.

‡ Technical assistance in this work was rendered by Miss Anita Cooper.

1 Libby, R. L., *J. Immunol.*, 1938, **34**, 71, 268.

2 Bukantz, S. C., and Bullowa, J. G. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 418.

3 Schmidt, L. H., and Hilles, C., *J. Inf. Dis.*, 1939, **65**, 273.

broth culture of the strain was injected intraperitoneally into an 18 g mouse. Twelve hours after the inoculation the mouse was sacrificed and a standard loop of heart's blood of the freshly killed animal was inoculated into 10 ml of blood broth. This cycle was repeated daily and used routinely for the preservation of the strain. After the eleventh and twelfth passages, the M.L.D. of the strain was found to be the same—8 organisms.§ Subsequent determinations have indicated that the virulence has not changed, even after the 50th passage. The 12-hour cultures contain a fairly constant number of organisms, usually between 50×10^6 and 100×10^6 per ml.

In the following experiments, all bacterial counts were made by the plating method with dilutions in broth. Separate pipettes were used for each dilution which was mixed 25 times before transfer. One ml of diluted culture, containing the desired inoculum, was seeded into each of a series of tubes containing 9 ml of 4% horse-blood-broth. A colony count of the original culture was performed, at the same time, in order to determine the size of inoculum used. All inoculated tubes were then incubated at 37°C for the duration of the experiment.‖ At 2, 4, 6, 8, 10, 12, 24, 28, 32, and 48-hour intervals, one tube was removed, a colony count performed and the contents of the tube filtered through the Swinney Seitz Filter Adapter.¶ All filtrates were sterile, and the pH, determined with nitrazine paper, was at least 7.0, even after a 48-hour period of incubation.** The concentration of capsular polysaccharide in each filtrate was then determined by titration of 1 ml of varying dilutions of each filtrate with 1 ml of a standardized serum in the zone of antibody excess; photronreflectometric measurements were made after incubation of each mixture in refraction cells for 20 minutes at 37°C. Dilutions of the filtrates were made in broth and the concentrations determined by reference to a standard curve prepared by titrating varying dilutions of the polysaccharide in broth, with the standard serum. Filtration control studies indicated that filtration of SSS in broth was not quantitative until adsorption by the filter had been completed. This adsorption occurred most rapidly in the presence of large numbers of bacteria (100,000,000 per ml) and the higher concentrations of SSS

§ According to the modification of the Park-Cooper technic employed by The Massachusetts Antitoxin and Vaccine Laboratory (White, *Biology of the Pneumococcus*, page 653).

‖ In preliminary experiments 4 tubes separately inoculated with the same number of pneumococci grew out an identical number of organisms in the same time.

¶ Becton-Dickinson, manufacturers, Rutherford, N. J.

** The original broth contained no glucose.

TABLE I.

Titration of Various Filtrates from Experiment II (9,000 Organisms per ml Inoculum) with Standard Serum III. Sensitivity of Photronreflectometer at 15.

Hour		Dilution of Filtrate in Broth. Und.	1-5	1-10	1-20	1-30	1-35	1-40	1-50	1-60	1-80	1-100	1-150	Avg SSS per ml
10	Corrected Photron Rdg.[1]				5.0	3.0		1.5	1.0					.013
	SSS Equiv. of Dilution[2]				.00075	.0004		Ind.[5]	Ind.					
	SSS Conc. in Orig. Filt.[3]				.015	.012								
12 and 50′	C. P. R.					8.5			5.0		3.0	2.0		.035
	SSS Eq. Dil.[2]					.00125			.00075		.0004			
	SSS Or. Fil.[3]					.037			.037		.032			
24	C. P. R.							20.0	16.0		8.0	7.0		.12
	SSS Eq. Dil.[2]							.0032	.0025		.0012	.001		
	SSS Or. Fil.[3]							.13	.13		.10	.10		
28	C. P. R.			17.5	24.0	24.0		21.0		15.5	12.5	8.0		.13
	SSS Eq. Dil.[2]			E.A.[4]	E.A.			.0033		.0023	.0019	.0012		
	SSS Or. Fil.[3]							.13		.14	.15	.12		
32	C. P. R.			14.0	24.0	23.0	20.5	21.0		13.0	10.0	6.5		.12
	SSS Eq. Dil.[2]			E.A.				.0033		.00195	.0015	.001		
	SSS Or. Fil.[3]							.13		.12	.12	.10		
48	C. P. R.			7.5	19.0	23.0	23.5	23.5	23.0		15.0	12.0	8.0	.18
	SSS Eq. Dil.[2]			E.A.	E.A.	E.A.			.0034		.0022	.0018	.0012	
	SSS Or. Fil.[3]								.17		.18	.18	.18	

[1] Corrected Photronreflectometer Readings—Obtained by subtracting the dry blank reading of the cell from the final turbidity reading.

[2] SSS (Capsular Polysaccharide) Equivalent of Dilution—Obtained by reference to a previously determined standard curve. This figure expresses the actual amount of SSS present in the 1 ml sample of the dilution titrated.

[3] SSS Concentration in the Original Filtrate—Obtained by multiplying SSS Equiv. of Dil. by the Dilution [*i.e.*, For 10 hr .00075 $\times$ 20 = .015] expressed as milligrams per milliliter.

[4] E.A.—Excess of antigen.

[5] Ind.—Indeterminate, because of inaccuracy in calculations of points on lowermost extrapolations of the standard curve.

(10-20 mg %). However, following a very large initial inoculum (900,000 per ml, Experiment III), low concentrations of SSS were obtained in early filtrates despite the presence of large numbers of bacteria (63,000,000 per ml), while later filtrates, obtained from cultures having approximately the same number of bacteria, yielded considerably higher concentrations of SSS. The influence of autolysis upon the increase of concentration of SSS has not yet been adequately investigated because of the difficulty in defining a technic for quantitative determination of the degree of autolysis. Judging from the rate of decrease of viable organisms, however, there is at least one period of significant increase in concentration of SSS in filtrates which appears to be unrelated to autolysis (13th to 24th hour, Experiment II) and more probably represents an alteration in the physiologic activity of the organisms.

A small quantity of capsular polysaccharide (0.5 mg %) was found after 48 hours, following an initial inoculum of 165 organisms per ml (Experiment I). A much larger quantity of polysaccharide (18 mg %) was found after an initial inoculum of 9,000 organisms per ml (Experiment II). Table I summarizes the titrations of some of the filtrates obtained following the inoculation of 9,000 organisms per ml. The remaining filtrates were titrated in the same way. Table II summarizes the results of this experiment in relation to the

TABLE II.

Experiment II. Growth of highly virulent pneumococcus III in 4% horse blood broth following an initial inoculum of 9,000 organisms per ml. Concentrations of SSS determined with the photronreflectometer.

Hrs.	Colony count	Log. of count	*No. of generations	†Generation time (min)	SSS concentration mg/100 ml
2	18,200	4.26	1.0	120	0
4′30″	384,500	5.59	4.4	34	0
6′40″	5,100,000	6.71	3.7	35	.18
8	24,060,000	7.38	2.2	36	.70
10	138,000,000	8.14	2.5	48	1.30
12′50″	170,000,000	8.23	0.3	566	3.5
24	105,000,000	8.02	—	—	12.0
28	75,000,000	7.88	—	—	13.0
32	47,500,000	7.68	—	—	12.0
48	40,000	4.60	—	—	18.0

* From formula for law of geometric progression

$$N = \frac{\text{Log. of Final Count} - \text{Log. of Initial Count}}{\text{Log. 2}}$$

† $\frac{\text{Time in minutes}}{\text{Number of generations}}$

After Chesney.[7]

[7] Chesney, A. M., *J. Exp. Med.*, 1916, **24**, 387.

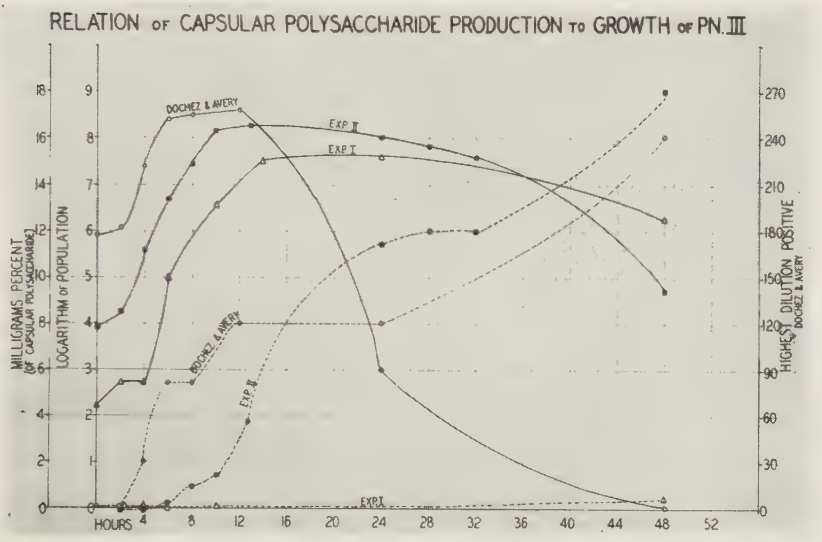

FIG. 1.

The relationship of increases in concentration of capsular polysaccharide III in culture filtrates to the phase of growth of Pneumococcus III in blood-broth; two different sizes of inoculation of an organism having the same virulence in each experiment.

Solid lines ———— Logarithm of populations.
Broken lines - - - - - - - - - Experiments I and II, Concentration of SSS in mg per 100 ml (mg%).
Broken lines - - - - - - - - - Dochez and Avery, highest dilution of SSS giving a positive reaction with the solution of antibody used by them.
Experiment I—Inoculum of 165 organisms per ml.
Experiment II—Inoculum of 9,000 organisms per ml.

growth-rate of the organisms. In Fig. 1 the logarithms of colony counts and mg % of polysaccharide produced are plotted, as ordinates, with time as abscissa.

Dochez and Avery[4] concluded, from their studies, that the largest quantity of capsular polysaccharide was found in those pneumococcal culture filtrates obtained during the phase of maximal growth of the organism. The present observations indicate that the greatest quantity of capsular polysaccharide is found in filtrates of blood-broth cultures obtained after the maximal growth phase is completed, *i. e.*, after the completion of the logarithmic phase of growth. In this regard it was interesting to plot the logarithms of the population and the polysaccharide production described in Dochez and Avery's protocol. (Polysaccharide concentration was plotted as the highest dilution giving a positive reaction, since a dilution method was em-

[4] Dochez, A. R., and Avery, O. T., (a) PROC. SOC. EXP. BIOL. AND MED., 1917, **14**, 126; (b) *J. Exp. Med.*, 1917, **26**, 477.

ployed by them in estimating polysaccharide produced.) These are also represented in Figure I; it is apparent that the major portion of polysaccharide in their experiment was found after completion of the logarithmic phase of growth. Of additional interest are the recent observations of Hoogerheide,[5] that the size of the capsule surrounding *Klebsiella pneumoniae (Bact. Friedlander)* begins to increase only after the completion of the logarithmic phase of growth, and the earlier observations of Cowan[6] that hemolysin appears in pneumococcal broth cultures only after completion of the logarithmic phase of growth.

Summary. The concentration of capsular polysaccharide appearing in blood-broth culture filtrates following the growth of a highly virulent, standardized strain of Pneumococcus III, was determined photronreflectometrically and correlated with the growth rate of the organism. An initial inoculum of 165 organisms per ml yielded a very small quantity of polysaccharide (0.5 mg %). An inoculum of 9,000 organisms per ml yielded 18 mg % of capsular polysaccharide in 48 hours. The greatest increases in concentration of polysaccharide occurred in two stages after the completion of the logarithmic phase of growth.

11264

Effect of Intravenous Injections of Yeast Extract on Spontaneous Breast Adenocarcinomas in Mice.

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In this laboratory, studies on the action of different spleen extracts on transplanted and on spontaneous tumors have been carried out for several years. It was shown in previous publications[1, 2] that the complete retrogression of transplanted Sarcoma 180 could be accomplished in 60% of the treated animals following subcutaneous injections of biologically[2] or chemically concentrated spleen

[5] Hoogerheide, J. C., *J. Bact.*, 1939, **38**, 367.

[6] Cowan, S. T., *J. Path. and Bact.*, 1934, **38**, 61.

[1] Lewisohn, R., *Surg., Gyn. and Obst.*, 1938, **66**, 563.

[2] Lewisohn, R., Leuchtenberger, R., and Laszlo, D., Internat. Cancer Congress, Atlantic City, Sept., 1939.

extracts. Furthermore, we succeeded in producing total regressions in spontaneous breast carcinomas in 27 mice by intravenous injections of spleen extracts.

This report deals with the action of another extract on spontaneous malignant tumors in mice, namely a yeast extract.

Nevorojkin[3] injected concentrated suspensions of yeast into transplanted tumors and noticed retardation of growth and, in some cases, regressions.

Maisin, Pourbaix and Caeymaex[4] demonstrated that they could retard the growth of experimental cancer in rabbits by adding boiled yeast to the food of the animals.

The yeast extract which was used by us for intravenous or subcutaneous therapy of malignant tumors was prepared in the following manner: 2500 g of Brewer's yeast is washed several times with distilled water. The yeast is boiled for 7 minutes with 9000 cc distilled water to which 0.9 cc of glacial acetic acid has been added. The extract is filtered through paper-pulp and concentrated *in vacuo* at 70°C to 1500 cc. It is precipitated with 1500 cc of absolute alcohol. The filtrate is concentrated *in vacuo* at 70°C to 900 cc.

The yeast extract was used intravenously, sometimes in combination with subcutaneous injections. The average dose for intravenous treatment was 0.1 cm, whereas 0.5 cc was injected subcutaneously. The intravenous injections were given into the tail of the mouse. When used subcutaneously, the extract was injected as far away from the site of the tumor as possible in order to avoid the possibility of direct action of the extract on the tumor. Daily, or on alternate days, injections were given until the tumor disappeared or were discontinued when the tumor did not respond to treatment. Injections of yeast extract were continued after disappearance of the tumor in order to avoid a possible recurrence.

Thirty-three tumor-bearing mice were used for these experiments. Twenty-two came from the Jackson Memorial Laboratory, Strain A. Eleven were bought from the Rockland Farm. They did not belong to a pure inbred strain.

The average size of the tumor at the onset of the treatment can be recognized from the sketches of the regressed tumors in Table I.

In each instance the malignant character of the tumor was established by biopsy before treatment was started. The histologic criteria of malignancy were chiefly cytologic, such as many atypical mitoses

[3] Nevorojkin, J., *Vestnik. Roentgenol.*, 1935, **15**, 344.

[4] Maisin, J., Pourbaix, Y., and Caeymaex, P., *Comptes rendus Soc. biol.*, Paris, 1938, **127**, 1477.

Table I. Effect of yeast extract on spontaneous breast adenocarcinomas in mice. J.M.L.=Jackson Memorial Laboratory. R.F.=Rockland Farms.

No.	Source	Actual size of tumor	Treatment started	Healed	Injections Intravenous	Injections Subcutaneous
310	J.M.L		Nov. 9, 1939	Nov. 25, 1939	11	0
350	"		" 10, "	Dec. 20, "	13	11
352	"		" 10, "	Jan. 9, 1940	13	14
362	"		" 10, "	Dec. 16, 1939	11	8
402	"	 2 separate tumors 1 anterior 1 lateral	Dec. 23, "	Jan. 13, 1940	11	10
404	"		" 23, "	" 16, "	12	10
R 18	R.F.		Jan. 13, "	" 22, "	7	7
R 22	"		" 15, "	" 21, "	5	4

and striking cell irregularities, because actual invasion of the adjacent healthy tissue could hardly be established in a biopsy which had to be made as small as possible.

Controls were first deemed unnecessary since it has generally been assumed and has been confirmed by personal communication from Dr. Little that breast carcinoma of Strain A Jackson Memorial Laboratory, proven by biopsy, never disappears spontaneously. However, we believed it was necessary to establish whether biopsy alone might not influence the natural evolution of this tumor. In 60 mice, Strain A Jackson Memorial Laboratory, and in 5 Rockland Farm mice, in which only biopsy was performed, we have not observed so far disappearance of the tumor (Strain A Jackson Memorial Laboratory). However, temporary regressions in size were noted.

Of 33 animals treated with yeast extract, the tumors disappeared in 8 mice and have not recurred so far (see table). Naturally, the animals remain under further observation. In 10 animals the tumor was reduced in size. In 15 instances either no change in the size of the tumor was noted or they had even increased in size when treatment was discontinued or the animals died.

Details of the treatment and the source of the 8 mice in which the tumors regressed following treatment with yeast extract are presented in Table I.

It is impossible in this brief presentation to describe in detail the

interesting microscopic changes which occur in tumors during the treatment with yeast extract. Extensive cell necrosis presents a very striking picture which will be described in a subsequent publication.

Furthermore, we postpone for a later date reports on the treatment of malignant tumors with yeast in powder form or in pellets.

The factors responsible for this action of yeast extract are of course entirely unclear. Certainly, many different possibilities must be considered. For instance, it remains to be determined whether bacterial factors which may have been present in yeast extracts were operative in the effect described. Duran-Reynals[5] observed total regressions of some spontaneous mammary carcinomas of mice due to repeated injections of bacterial filtrates.

Summary. In 8 out of 33 treated mice complete regressions of spontaneous breast adenocarcinomas were effected with intravenous and subcutaneous injections of yeast extract.

11265 P

Action of Sulfathiazole and Sulfamethylthiazole on *Staphylococcus aureus.*

GEOFFREY RAKE AND C. M. MCKEE.

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Studies[1–4] on certain thiazole analogues of sulfapyridine[5, 6] have indicated that these drugs have a therapeutic activity equal to that of sulfapyridine when tested against pneumococci, streptococci, meningococci and the agent of lymphogranuloma venereum; and that the toxicity of sulfathiazole itself is usually less than that of sulfapyridine. More recently there have appeared several communications dealing with the activity of sulfathiazole and certain of its derivatives

5 Duran-Reynals, F., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1517.

1 van Dyke, H. B., Greep, R. O., Rake, Geoffrey, and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 410.

2 McKee, C. M., Rake, Geoffrey, Greep, R. O., and van Dyke, H. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 417.

3 Rake, Geoffrey, van Dyke, H. B., Corwin, W. C., McKee, C. M., and Greep, R. O., *J. Bact.*, 1940, **39**, 45.

4 Cooper, F. B., Gross, Paul, and Lewis, Marion, PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 421.

5 Fosbinder, R. J., and Walter, L. A., *J. Am. Chem. Soc.*, 1939, **61**, 2032.

6 Lott, W. A., and Bergeim, Frank H., *J. Am. Chem. Soc.*, 1939, **61**, 3593.

on staphylococci.[7–10] Herrell and Brown[7] found much greater *in vitro* bacteriostatic activity of sulfamethylthiazole than of sulfathiazole against *Staphylococcus aureus.* However, they give only one experiment and that in brief. They also state that "much more marked protection" was accorded mice infected with *Staphylococcus aureus* when these animals were treated with sulfamethylthiazole than when treated with sulfathiazole, but give no details. Lawrence[8] found that sulfamethylthiazole had a greater bacteriostatic activity than had sulfathiazole but his results would seem to be in part vitiated by the use of supersaturated solutions. Barlow and Homburger,[9] using small numbers of mice, give results which indicate little if any greater therapeutic activity of sulfamethylthiazole as compared with sulfathiazole. Finally, Helmholz[10] found that voided urine from patients treated with either of these two drugs was markedly bactericidal for *Staphylococcus aureus* even with drug concentrations as low as 16 mg %.

In carrying out *in vitro* studies with sulfathiazole, sulfamethylthiazole and sulfapyridine it has been found that minor variations in the technic used or in the broth menstruum have greatly influenced results. When, however, concentrations of drug of approximately 200 mg % are used, and supersaturated solutions are avoided, it has been found that the methyl derivative has greater bacteriostatic activity than sulfathiazole which, in turn, is more active than sulfapyridine. Table I shows such an experiment.

Poured plates inoculated with 0.1 ml of different dilutions of the

TABLE I.

Action of Sulfathiazole (ST), Sulfapyridine (SP), and Sulfamethylthiazole (SMT) on *Staphylococcus aureus in vitro.*

	ST	SP	SMT	Control
Immediate	40*	40	80	20
2 hr	80	50	140	230
4 "	170	1170	140	7100
6 "	1800	9200	1300	530,000
24 "	460 million	1260 million	7.4 million	1580 million

Drug concentrations in each case 198 mg% added to broth after autoclaving.
*Figures indicate number of colonies per ml.
Inoculum was 0.1 ml of 10^{-6} dilution of 18-hr culture of strain MK in 2 ml volume.

7 Herrell, W. E., and Brown, A. E., *Proc. Staff Meetings Mayo Clinic,* 1939, **14**, 753.

8 Lawrence, C. A., (a) *J. Bact.,* 1940, **39**, 46; (b) Proc. Soc. Exp. Biol. and Med., 1940, **43**, 92.

9 Barlow, O. W., and Homburger, E., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 792.

10 Helmholz, H. F., *Proc. Staff Meetings Mayo Clinic,* 1940, **15**, 65.

cultures were made at the time intervals indicated. Counts were made and those recorded are from plates which gave the largest number of easily countable colonies.

Other experiments under similar conditions but with colony counts at different intervals of time confirm the relationship of activity, namely, sulfamethylthiazole > sulfathiazole > sulfapyridine. In certain cases, however, sulfapyridine and more particularly sulfamethylthiazole have not remained in solution during the test presumably because the starting solution was supersaturated. It would seem essential to avoid such precipitation of drug which may vitiate the results by decreasing the total amount of drug in solution. This condition of supersaturation seems to be rather a factor of the constitution of the broth than of the actual concentration of drug. Other factors, such as autoclaving the drug in the broth (with or without added glucose) or adding the drugs to the broth after the latter has been autoclaved (as in Table I), have also altered the results.

The agar cup plate method of testing *in vitro* activity[11] was also used.* When the drugs were mixed with 5% gum acacia, sulfathiazole was found to be the most active compound producing inhibition of growth in a dilution of 1:8000 as compared to 1:3000 for sulfamethylthiazole and 1:1000 for sulfapyridine. Differences in solubility and diffusibility probably account for these differences in activity.

In the *in vivo* experiments drugs were administered to mice in 1% of their normal dry diet.[2] Five different strains of *Staphylococcus aureus*, 3 hemolytic and 2 non-hemolytic, were used. The mice were inoculated intraperitoneally and received in each case approximately 500,000 organisms suspended in 1 ml of 5% mucin. Mice were kept on the drugs for 10 days and then observed for an additional 21 days after return to normal diet. At the end of the 31-day period survivors were autopsied and the persistence of infection noted by obtaining positive cultures from one or all of the following organs: spleen, liver and kidney. Deaths after 7 days were very unusual.

As seen in Table II, sulfapyridine had very little if any activity. Both sulfathiazole and sulfamethylthiazole protected mice and the former drug was slightly, but consistently better than the latter, as shown both by the number of surviving mice and by the percentage of residual infection in the organs as determined at autopsy. It may be noted that rough and other variants were found among the strains of

11 Ruehle, G. L. A., and Brewer, C. M., U. S. Dept. of Agriculture, 1931, circular No. 198.

* We wish to express our thanks to Dr. Brandt Rose of Philadelphia for suggesting the use of this test.

TABLE II.
Action of Sulfathiazole, Sulfapyridine and Sulfamethylthiazole *in vivo*.

Strain	No. of mice in each group	ST	SP	SMT	Control
NH MK	30	22*	3	22	0
NH Co.	40	17	4	14	3
H 469	30	15	2	9	2
H 687	30	21	3	15	1
H 631	20	20	6	18	2
Total	150	95	18	78	8
% of survivors whose organs gave positive cultures		14%	18%	25%	12.5%

H—Hemolytic strain. NH—Non-hemolytic strain.
* Figures indicate number of survivors over 31-day period.

Staphylococcus aureus recovered from the mice treated with each of the 3 drugs.

Summary. In *in vitro* experiments with *Staphylococcus aureus* sulfamethylthiazole has shown greater bacteriostatic activity than sulfathiazole; the activity of the latter was in turn greater than that of sulfapyridine. In *in vivo* experiments with the same organism sulfapyridine has had little, if any, activity. Both sulfathiazole and sulfamethylthiazole have protected mice and the former drug has been slightly but consistently more active than the latter.

11266

Mechanism of the Blood Pressure Response to Anoxia During Hypoglycemia.

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Gellhorn, Ingraham and Moldavsky[1] found that breathing a mixture of 6.2% oxygen in nitrogen caused a marked rise in blood pressure in dogs which had been made hypoglycemic by the injection of insulin. Intravenous injection of glucose made this blood pressure

[1] Gellhorn, E., Ingraham, R. C., and Moldavsky, L., *J. Neurophysiol.*, 1938, **1**, 301.

response disappear. Since, according to Ingraham and Gellhorn,[2] this rise in blood pressure is not due to a discharge of adrenalin, the authors first mentioned assumed that this phenomenon is caused by a "tremendous excitation of the sympathetic centers." Lambert and Gellhorn[3] reported (contrary to Heymans, Nowak and Samaan[4]) that the rise in blood pressure caused by the inhalation of a gas mixture low in oxygen is not due to a direct stimulation of the medullary centers but to a stimulation of the chemoreceptors in the carotid sinus and in the thorax, causing through a reflex the rise in blood pressure. Therefore, Gellhorn, Ingraham and Moldavsky think it probable that the responsiveness of the medullary centers to these impulses is increased by hypoglycemia.*

The same phenomena have been observed in man by Kraines and Gellhorn[5] under similar conditions, and Gellhorn[6] has used the above assumptions to explain the therapeutic effect of insulin shock in cases of schizophrenia.

When trying to explain the large rise in blood pressure from anoxia during hypoglycemia it should be kept in mind that the blood pressure is the resultant of a large number of often opposite reactions. Heymans and Bouckaert[7] emphasized that the excitability of the central nervous system (c.n.s.) is an important factor in determining the result of anoxia on the blood pressure.

To investigate the influence of the excitability of the c.n.s. on the blood pressure response to anoxia, this reaction was studied at various levels of narcosis. The blood pressure was recorded with a mercury manometer connected with one of the carotid arteries. The dye "fastusol BBA" served as an anticoagulant.[8] In very superficial narcosis the blood pressure response of dogs, cats and rabbits to breathing nitrogen or a gas mixture of 4.7% oxygen in nitrogen for 1 to 2 minutes (either spontaneously or by artificial respiration) was inconsistent; sometimes a small rise resulted, but in other experiments an appreciable fall of the blood pressure was observed. In all experiments deepening of narcosis (chloralose or nembutal) increased

2 Ingraham, R. C., and Gellhorn, E., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 315.

3 Lambert, E., and Gellhorn, E., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 427.

4 Heymans, C., Nowak, S. J. G., and Samaan, A., *Compt. Rend. Soc. Biol.*, 1934, **117**, 248.

* Assuming that the response of the chemoreceptors to anoxia is the same in the hypoglycemic and in the normal state.

5 Kraines, S. H., and Gellhorn, E., *Am. J. Psychiatry*, 1939, **95**, 1067.

6 Gellhorn, E., *Arch. Neurol. Psychiat.*, Chicago, 1938, **40**, 125.

7 Heymans, C., and Bouckaert, J. J., *Ergebn. Physiol.*, 1939, **41**, 28.

8 Modell, W., *Science*, 1939, **89**, 349.

the rise in blood pressure as a response to anoxia, or changed the fall of the pressure during anoxia into a rise. As narcosis became deeper the blood pressure level often fell, so that the blood pressure rise as a response to anoxia, though larger, often failed to reach as great a height as a similar rise during light narcosis. In Fig. 1 are shown 3 responses to anoxia in an experiment which does not have this disadvantage. A cat of about 3 kg body weight was given artificial respiration and at 10-minute intervals the animal was caused to breathe nitrogen instead of air for a period of 1 minute. The response in very light narcosis (100 mg chloralose) was a very marked fall of the blood pressure accompanied by a decrease of the heart rate (A). Three previous periods of anoxia in this state of narcosis had given almost identical curves. After injecting another 100 mg of chloralose the blood pressure response (B) consisted of a rise followed by a fall. In curve A also, a suggestion of this initial rise can be seen. After the injection of another 100 mg of chloralose the initial rise was still larger and the following fall in blood pressure had diminished materially (C). The blood pressure was practically the same at these 3 levels of narcosis. These experiments suggest that a moderate depression of the functions of the c.n.s. favors a blood pressure rise as a response to anoxia. It is likely that this is caused by a suppression by the narcosis of reactions counteracting a marked rise in blood pressure, either caused directly by the anoxia or indirectly by a slight rise in blood pressure (*e. g.*, the carotid sinus pressure reflex). It has to be assumed that these latter reactions are depressed more markedly by the narcosis than are the reactions causing the rise in blood pressure.

It is well known that hypoglycemia causes a depression of the functions of the c.n.s., which can in severe conditions lead to coma. Less severe hypoglycemia also depresses the functions of the c.n.s. as has been shown by Wiedeking[9] for cortical functions. It is therefore quite possible that the combined effects of hypoglycemia and narcosis caused, in the experiments of Gellhorn, Ingraham and Moldavsky, a depression of the c.n.s. to such a degree that anoxia brought about a marked rise in blood pressure because the reactions counteracting this rise were depressed. To examine this conception the effect of hypoglycemia on one of the reactions counteracting a marked rise in blood pressure was investigated, namely the depressor reflex in the rabbit. Two to 3 hours after the intravenous injection of 10 to 15 units of insulin per kg body weight in the fasting animal, the depressor nerve was isolated and the carotid artery arranged for

[9] Wiedeking, J., *Z. ges. Neurol.*, 1937, **159**, 417.

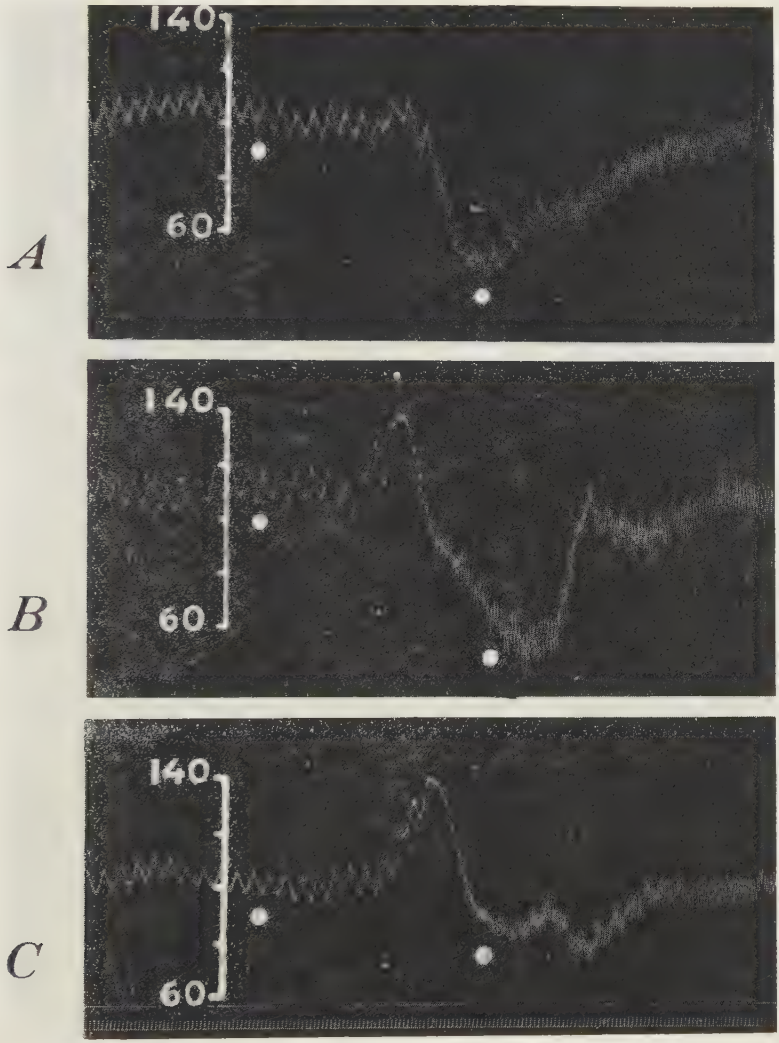

Fig. 1.

Three blood pressure responses to anoxia (nitrogen for 1 minute) at 3 levels of narcosis in a cat of 3 kg bodyweight. The white dots indicate the beginning and end of introducing nitrogen into the apparatus for artificial respiration. "A" after administering 100 mg of chloralose, "B" after a total of 200 mg, and "C" of 300 mg of chloralose. Time intervals, 1 second.

blood pressure recording. The animals usually were in a hypoglycemic coma and showed occasional convulsions. Chloralose or nembutal was used as narcotic. The blood sugar was determined before and after the intravenous injection of 3 to 5 gr glucose. The depressor nerve was stimulated faradically with maximal stimuli.

TABLE I.

Exp.	Blood pressure level and fall in blood pressure during 10 sec stimulation of the depressor nerve						Blood sugar in mg/100 cc	
	Before glucose		After glucose					
			10 min		20 min			
	B.p. level	B.p. fall	B.p. level	B.p. fall	B.p. level	B.p. fall	Before glucose	After glucose
1	80	21	86	28	88	34	30	197
2	76	0	80	4	46	4	—	—
3	64	16	68	26	60	28	41	249
4	72	9	74	14	76	26	30	215
5	58	0	56	11	60	16	48	225
6	—	3	—	4	—	6	—	—
7	76	3	80	13	80	14	—	—
8	88	3	84	11	88	12	30	152

Before injecting glucose the depressor nerve was stimulated repeatedly; no large fluctuations of the responses were observed. In all the cases presented in Table I the fall in blood pressure during 10 sec of depressor stimulation was larger after administering glucose than during hypoglycemia. It takes about 20 minutes for this effect of the glucose injection to fully develop. In some cases (2 and 5) the stimulus did not cause any reaction during hypoglycemia; after injection of glucose, however, the same stimulus caused an appreciable fall. It can be concluded that this reflex, like many other reactions of the c.n.s., can be depressed by hypoglycemia.

On repeating Gellhorn, Ingraham and Moldavsky's experiment on the rabbit we found in part of the cases no marked blood pressure rise caused by anoxia, though the blood sugar level was found to be quite low. It is possible that this is characteristic for the rabbit, since Gellhorn, Ingraham and Moldavsky, working on the dog, did not report this inconstancy. It must be assumed that in these cases the combined effect of hypoglycemia and narcosis did not depress markedly the reactions counteracting a blood pressure rise. In agreement with this the depressing effect of hypoglycemia on the depressor reflex was found only in part of the experiments, in the others the injection of glucose in the hypoglycemic animal caused no change in this reflex.

This explanation of the marked rise in blood pressure as a response to anoxia during hypoglycemia, as a depression of the functions of the c.n.s. is quite in keeping with the usual depressing effect of hypoglycemia on the c.n.s.

Conclusion. The large blood pressure rise due to anoxia, observed during hypoglycemia, may be caused by a severe depression by the combined effect of narcosis and hypoglycemia of those reactions which counteract a marked rise in blood pressure, the reactions causing this rise in blood pressure being less severely depressed.

11267 P

Bleeding Induced in Uterine Mucosa of the Rabbit by Estrogenic Hormone.

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By using gonadotropic hormone it is possible to produce in the rabbit a profuse bleeding which roots up the entire uterine mucosa.[1] The blood penetrates through the lifted areas of the epithelium into the uterine cavity and finally escapes into the vagina. This bleeding is observed especially after intravenous administration of the hormone and only rarely after subcutaneous administration. The most suitable dosage proved to be 100 RU gonadotropic hormone daily for 5 days. The bleeding appears on the 6th day. This effect was obtained with gonadotropic hormone from pregnancy urine or pregnant mare serum. The immature rabbit, weighing 1000-1400 g, is more suited for these experiments than when sexually mature. If the extirpated uterus is inspected against a strong light the blood can be recognized in the uterus with the naked eye but the macroscopical findings were not solely relied upon. Each preparation was examined histologically. In some cases the blood was found distributed over wide areas, sometimes there were only circumscribed spots. To be sure that bleeding had not been artificially produced by handling, the abdomen was opened, then filled with 4% formalin and the uterus was removed several hours later. It was possible to induce proliferation, and in some cases, progestational transformation in the mucosa uteri by administration of gonadotropic hormone for 5 days. In no instance was the bleeding found in the progestationally altered mucosa but only in the proliferative one. In the same uterine horn different areas may show different stages of development. Even in such cases the bleeding was always found in the proliferatively developed areas. From this we may deduce that progesterone counteracts the bleeding effect.

Bleeding could not be obtained in the castrated immature animal by the use of gonadotropic hormone, so it was obvious that the effect was produced *via* the ovary. The question now arose which of the two ovarian hormones was responsible for the production of the bleeding. The corpus luteum hormone could be eliminated, since no bleeding was ever encountered in the progestational mucous mem-

[1] Zondek, B., *J. of Obstr. and Gynæcol. of the Brit. Emp.*, 1938, **45**, 1.

brane; and experiments conducted with progesterone had also negative results. It is, therefore, the estrogenic hormone which should be considered in this connection. We injected into immature rabbits 100 IU of estrone in aqueous solution daily for 5 days. There was in some cases slight circumscribed bleeding, but we did not find the marked bleeding which spreads under the entire mucosa, eventually penetrating through the epithelium into the cavum uteri, as with gonadotropic hormone. Since it was not possible to produce bleeding of the uterine mucosa with either of the two ovarian hormones (estrone, progesterone) we suggested a third ovarian factor, hitherto unknown, which might be stimulated by gonadotropic hormone.[1] This assumption was found unnecessary, for it has been found possible to achieve the bleeding effect with estrogenic hormone, if a special experimental procedure is adhered to, taking into account the following factors:

(1) Dosage.
(2) Interval between administration of hormone and examination for the bleeding effect.
(3) Mode of application.

The most suitable dosage was 2 injections of 500 to 750 IU of estrone given at intervals of 12-24 hours. It is of prime importance that a powerful hormonal stimulus should be given in the course of one day, then wait several days. Usually the bleeding appears after an interval of 5 days. The most effective mode of application is by the intravenous route. The intravenous injection of estrogenic hormone is practicable in aqueous solution prepared in the following way: Dissolve 10 mg of crystallized estrone in a small quantity of absolute alcohol, add 1 cc of n/NaOH solution, after some time add water to 100 cc, so that the estrone is dissolved in n/100 NaOH. The alcohol is then evaporated *in vacuo*. One cc of this solution contains 1000 IU of estrone.

The experiments were performed with 13 immature rabbits. When estrone was injected subcutaneously in oily solution hyperaemia, but no bleeding, was observed (R. 604 and 606). When the hormone was spread over 4 days (R. 591) or when too large doses were used (3000 IU) bleeding was not obtained (R. 576, 579, 597). Adhering to the above described experimental procedure bleeding appeared in 5 of the experimental animals (Table I).

The estrone-produced bleeding is identical with that produced by means of gonadotropic hormone, yet does not seem to occur so regularly. The vessels show lacuna-like dilation, the blood penetrates the mucosa, lifts the epithelium and enters the uterine cavity and

TABLE I.
Bleeding of the Uterine Mucosa of the Rabbit Induced by Estrogenic Hormone.

Animal (rabbit) No.	Preparation	Dosage (IU)	Interval between injections	Mode of administration	Bleeding	Day when uteri were examined
565	Estrone aqu.	2x750	1 day	i.v.	+	4
566	"	2x750	1 "	i.v.	+	5
569	"	2x750	1 "	i.v.	+	4
588	"	2x750	1 "	i.v.	+	5
589	"	2x750	1 "	i.v.	+	5
605	Estrone ol.	2x500	1 "	s.c.	+	5
567	Estrone aqu.	1x750	—	i.v.	—	3
576	"	3x1000	18 hr	i.v.	—	5
579	"	3x1000	18 "	i.v.	—	5
591	"	4x300	4 days	i.v.	—	5
597	"	3x1000	18 hr	i.v.	—	5
604	Estrone ol.	2x250	1 day	s.c.	Hyperemia	5
606	"	2x750	1 "	s.c.	"	5

vagina. The anatomical picture greatly resembles that found in bleedings from a proliferatively developed mucosa in humans.

The sexual cycle, in the rabbit, is without bleeding. That it is possible to produce bleeding as in humans and monkeys, may provide the opportunity of studying the mechanism of the uterine bleeding in rabbits.

Summary. With estrone bleeding may be induced in the uterine mucosa of the rabbit. The blood penetrates through the lifted epithelial areas and eventually enters the uterine cavity and vagina. This effect may be obtained by two injections of 500 to 750 IU of estrone given intravenously during 12 or 24 hours. Bleeding appears after 4-5 days.

11268

Anterior Pituitary Extracts and the Brunn Reaction in Frogs.

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When pharmacopoeial extracts of the "posterior pituitary gland" are injected into frogs immersed in water there follows an increase in body weight due to an uptake of water which lasts 3 to 6 hours at room temperature. This reaction was first investigated by Fritz Brunn at Prague[1] and we shall refer to it simply as the Brunn reac-

[1] Brunn, F., *Zeit. f. exp. Med.*, 1921, **25**, 170.

tion. It has been shown[2] that the uptake of water in the Brunn reaction is 2 to 3 times as great in the summer as in the winter months in this locality. No exogenous factor was found to account for this seasonal variation which was tentatively ascribed to variation in some endogenous mechanism.[2]

Zahl[3] has reported that there is a seasonal variation in the histological picture of the pars distalis of the frog with more acidophilic cells in the winter than in the summer. The acidophilic cells of the buccal lobe are believed to elaborate growth hormone and possibly other hormones such as the thyrotropic and gonadotropic factors.[4, 5] We therefore considered it possible that an excessive production of anterior pituitary principles might have accounted for the depression of the Brunn reaction in our winter frogs. That anterior and posterior pituitary hormones antagonize each other upon certain phases of mammalian water balance has been known since the initial experiments of Harvey Cushing and his colleagues, von Hann and others.[6] Elmer writing from Lwöw quotes Carter as stating that a "Ringer extract of the whole pituitary . . . . produced the winter condition in a summer heart" of frogs.[7] Biasotti[8] found that an extract of the anterior part of the pituitary body had no immediate effect on the body water of a South American amphibian, *Leptodactylus ocellatus.*

If anterior pituitary hormones account for the winter depression of the Brunn reaction, they may act in one or both of two ways, either by directly neutralizing the water balance component of pituitary (posterior lobe) extract or by so altering the receptor cells upon which this extract acts that it cannot effect as great an uptake of water into the body. We wish to report experiments bearing upon the first possibility. Using the same technic as in previous studies of the Brunn reaction,[2] we injected into frogs in water pituitary (posterior lobe) extract without and with several commercial anterior pituitary, human pregnancy urine and pregnant mare serum preparations, the latter in doses corresponding by weight to 1 to 1000 times

2 Boyd, E. M., Mack, E. G., and Smith, A. E., *Am. J. Physiol.*, 1939, **127**, 328.

3 Zahl, P. A., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 56.

4 Van Dyke, H. B., *The Physiology and Pharmacology of the Pituitary Body*, Univ. of Chicago Press, 1936, and Vol. 2, 1939.

5 Symposium: *The Pituitary Gland*, Williams and Wilkins, Baltimore, 1938.

6 Fisher, C., Ingram, W. R., and Ranson, S. W., *Diabetes Insipidus and the Neurohormonal Control of Water Balance*, Edwards Bros., Ann Arbor, 1938.

7 Elmer, A. W., *Iodine Metabolism and Thyroid Function*, Oxford Univ. Press, London, 1938.

8 Biasotti, A., *Comp. rend. Soc. de biol.*, 1923, **88**, 361.

the average recommended single injected human dose. These preparations used included Gonan and Serogan (B.D.H.), Antophysin (Winthrop), Antuitrin-G (P.D.&Co.) and the gonadotropic, thyrotropic and lactogenic fractions marketed by Ayerst, all of these preparations being generously provided us by the companies concerned. A grant which defrayed part of the expenses of the investigation was provided by Parke, Davis and Co. through Dr. E. A. Sharp.

None of these preparations had any effect upon the Brunn reaction in the doses employed. To illustrate this, the percentage uptake of water was averaged in all groups of frogs receiving the same human dose equivalent of the preparations and 1 international unit of pituitary (posterior lobe) extract per 10 g body weight, giving a total of 96 frogs to each group and the mean values so obtained have been compiled in Table I. Similar experiments made with lower doses of pituitary (posterior lobe) extract down to 0.1 units per 10 g yielded similar negative results. These results indicate that that fraction of pituitary (posterior lobe) extract which produces the Brunn reaction is not directly neutralized by any of the anterior pituitary or anterior pituitary-like hormones present in the preparations used.

It seemed unlikely that any of these anterior pituitary or anterior pituitary-like preparations could of themselves affect body water during the 5-hour interval after their injection when body weight was being measured. To be sure of this, we injected them without pituitary (posterior lobe) extract and in a dosage range similar to that used above and found in some 500 frogs that changes in body weight were not significantly different from those in controls.

Conclusion. A number of anterior pituitary and anterior pituitary-like preparations were found to have no effect during 5 hours after their injection into frogs in water either on normal body water or upon the Brunn reaction.

TABLE I.

Uptake of Water by Leopard Frogs in Water Injected with Pituitary (Posterior Lobe) Extract with and without Increasing Doses of Anterior Pituitary Preparations (AP).

Group	% uptake of water (Mean 96 frogs)			
	2 hr	3 hr	4 hr	5 hr
Pituitrin alone	14.1	15.6	13.5	9.9
" + equivalent human dose AP	13.1	15.4	13.1	9.9
" + 10X equivalent human dose AP	11.8	15.2	13.8	10.4
" + 100X " " " "	10.0	14.4	12.8	9.4
" + 1000X " " " "	10.3	15.1	13.5	10.8

11269

Studies on the Bovine Electrocardiogram. I. Electrocardiographic Changes in Calves on Low Potassium Rations.*

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Four calves were placed on a semipurified ration which analyzed 0.10-0.12% potassium. Four control calves were given the same ration except that potassium was added to bring the level of this constituent to 0.35%. The animals were placed on this ration at 160 days of age. Previous to this they received whole milk and a small amount of hay and grain.

Electrocardiograms were usually taken at monthly intervals, but for some periods more frequently. The electrodes, which were of the German silver plate type, were placed on the upper part of the 2 fore limbs and just above the hock on the left hind leg. The areas on which the electrodes were placed were clipped, cleaned and covered with electrode paste applied with vigorous rubbing.

Serum potassium determinations were made on all calves at 2-week intervals, more often when deemed necessary, by a modification of the titrimetric method of Shohl and Bennett.[1]

Plasma calcium, phosphorus, and magnesium determinations were carried out at weekly intervals in order to make certain that the ration was not deficient in these elements.

Blood Changes. Calf C 394. On the 270th day of the experimental period this calf had a serum potassium value of 17.0 mg %. Seventy days later this value had fallen to 10.2 mg % and for a period of about 60 days it remained below 13.5 mg %. On the 350th day of the experiment the potassium in the ration was increased to 0.20% for a period of 130 days after which the potassium was again reduced. During this 130-day period the serum potassium level increased to 18.0 mg %, and it has since varied between 15 and 18 mg % in spite of the reduction of potassium in the ration.

Calf C 395. On the 270th day of the experimental period the serum potassium level of this calf was 21.9 mg %. On the 340th day it had decreased to 11.3 mg % and it remained below 14.0 mg % for a period of about 60 days. The potassium of the ration was then

* Published with the authorization of the Director of the Michigan Agricultural Experiment Station as journal article No. 406 (n.s.).

1 Shohl, A. T., and Bennett, H. B., *J. Biol. Chem.*, 1928, **78**, 643.

increased to 0.20%. The serum potassium promptly increased to 19.5 mg % and had reached a level of 22.4 mg % when the animal was slaughtered.

Calf C 409. From a level of 19.5 mg % on the 75th day of the experimental period the serum potassium decreased to 10.7 mg % on the 140th day. A low level was maintained for about 90 days. On the 175th day the potassium of the ration was increased to 0.20% and it was kept at this level for 130 days. During this time the serum potassium values returned to normal and have remained so in spite of the reduction of the potassium in the ration to 0.10% at the end of the 130-day period.

Calf C 410. The changes in this calf closely paralleled those of C 409. The serum potassium value of 17.6 mg % on the 70th day decreased to 13.8 mg % on the 160th day. Low values were maintained for about 60 days. During the 130-day period in which the potassium of the ration was increased to 0.20%, the serum potassium rose to 21.0 mg % and has since remained at or near this level.

The serum potassium values of these 4 calves are shown graphically in Fig. 1.

The serum potassium values for the 4 control calves averaged respectively 21.4 ± 1.2 mg %, 20.4 ± 1.5 mg %, 20.5 ± 1.4 mg % and 21.6 ± 1.8 mg %.

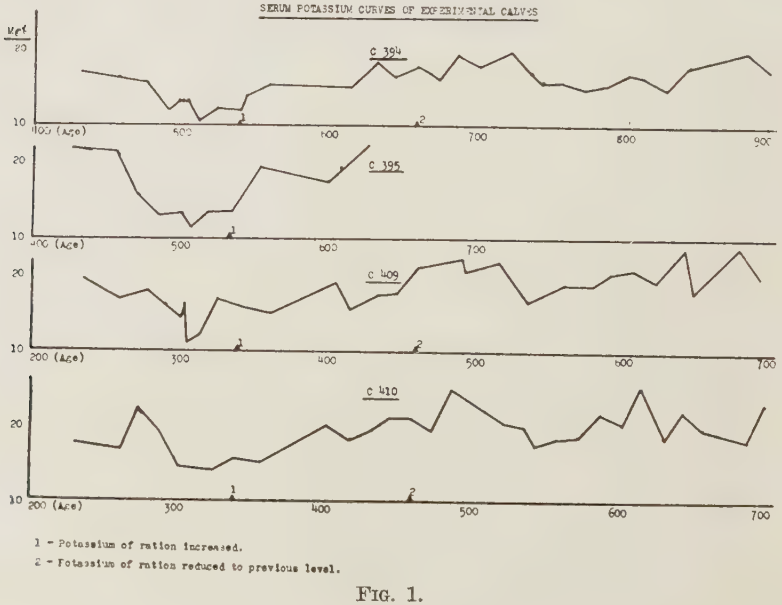

FIG. 1.
Serum potassium curves of the experimental calves.

The calcium, phosphorus and magnesium values of the plasma remained within normal limits in both experimental and control animals.

Electrocardiographic Changes. Three of the 4 experimental animals showed pronounced changes in the electrocardiogram. The other (C 410) failed to show any change, possibly because the serum potassium did not fall so low or remain at as low a level for so long a period in this calf as in the others.

The outstanding change in the electrocardiograms of these calves consisted in a pronounced increase in the duration of the QRS. The Q-T interval, also, increased, but this increase was obviously due mainly to the broadened QRS deflection. The P-R interval was within normal limits at all times. In 2 calves the QRS interval increased to more than 0.16 second and in one calf it exceeded 0.20 second. These QRS intervals are approximately twice as long as those displayed by the electrocardiograms of normal mature cattle and the electrocardiograms of the control animals. In a group of 97 animals, 60 of which were over 2 years of age, the average duration of QRS was .094 second and its maximum duration was 0.12 second (Alfredson, unpublished data). Barnes, Davis, and McKay,[2] who studied

TABLE I.
Heart Rate and Duration of QRS in Experimental Calves.

C 394			C 395			C 409			C 410		
Age	Rate	QRS	Age	Rate	QRS	Age	Rate	QRS	Age	Rate	QRS
175	43	.10	177	72	.08	26	107	.06	32	120	.06
216	42	.12	210	44	.10	77	75	.06	83	100	.06
253	58	.08	255	42	.08	105	84	.06	111	94	.06
260	65	.09	304	125	.06	176	112	.07	172	94	.06
309	79	.12	305	107	.08	189	88	.07	185	107	.08
330	100	.12	381	100	.10	211	88	.08	207	79	.08
386	79	.10	403	84	.12	224	100	.06	220	88	.08
408	84	.10	416	63	.11	237	88	.08	233	63	.08
421	125	.10	429	50	.12	299	63	.14	272	60	.07
434	52	.10	461	48	.13	300	88	.14	295	43	.08
460	47	.10	482	52	.12	301	92	.12	309	43	.08
484	65	.10	505	50	.16	302	88	.08	359	68	.08
485	65	.08	555	47	.16	306	77	.08	437	72	.08
486	65	.10	633	84	.18	308	79	.10	479	50	.08
490	56	.11				313	42	.09	528	75	.09
492	56	.12				363	72	.12	563	72	.09
497	33	.12				441	57	.12	595	75	.08
545	52	.12				483	64	.19	623	72	.10
623	75	.17				532	79	.14			
665	63	.18				557	68	.16			
714	63	.20				591	70	.15			
749	56	.24				619	60	.18			
773	48	.20									
801	50	.24									

2 Barnes, L. K., Davis, G. K., and McKay, C. M., *Cornell Vet.*, 1938, **38**, 16.

TABLE II.
Heart Rate and Duration of QRS in Control Calves.

C 387			C 392			C 419			C 420		
Age	Rate	QRS	Age	Rate	QRS	Age	Rate	QRS	Age	Rate	QRS
217	50	.06	214	72	.08	56	150	.06	67	54	.06
247	94	.06	219	54	.07	69	75	.06	90	58	.07
298	68	.06	250	88	.07	114	55	.07	120	94	.07
340	68	.07	286	91	.08	135	68	.07	154	65	.07
361	57	.08	293	97	.08	174	75	.07	232	68	.07
417	58	.09	300	100	.08	208	120	.08	274	100	.08
439	68	.07	342	75	.08	286	88	.08	323	88	.08
454	75	.08	363	68	.08	318	100	.07	358	88	.08
506	60	.07	398	75	.09	367	115	.08	392	84	.07
529	63	.10	419	68	.08	402	65	.07	420	79	.08
559	54	.08	441	65	.09	436	84	.08	517	72	.08
671	56	.11	467	65	.08	464	88	.08	558	79	.08
713	65	.10	506	68	.09	561	65	.09			
763	57	.12	542	60	.09	602	72	.09			
			569	60	.08						
			604	42	.09						
			682	72	.08						
			724	94	.09						
			773	63	.10						

normal calves ranging from 12-589 days of age, reported QRS intervals similar in length to those observed by Alfredson.

The changes in duration of the QRS complex and the heart rate are shown in Table I. Table II gives similar data for the 4 control calves.

The increase in duration of QRS began in 2 of the calves before the lowest levels of serum potassium were reached. In the third calf the increase began to appear when the lowest blood values were observed. The intervals became progressively greater and did not subsequently decrease even though the serum potassium values returned to approximately normal levels when the potassium content of the ration was raised.

The increase in the duration of the QRS complex was accompanied by equally striking changes in its contour and voltage. These changes are illustrated in Fig. 2, in which the electrocardiograms of calf C 394 are reproduced. The changes which occurred in the other animals were similar in a general way but varied in detail. It will be noted that the changes were progressive, and that the final record[4] resembles the human and canine curves which depict left bundle-branch block. It has been shown, however,[3] that section of the left branch of the His bundle in the calf produces relatively minor changes in the QRS interval and curves here in question cannot, therefore, be

[3] Alfredson, B. V., and Sykes, J. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 580.

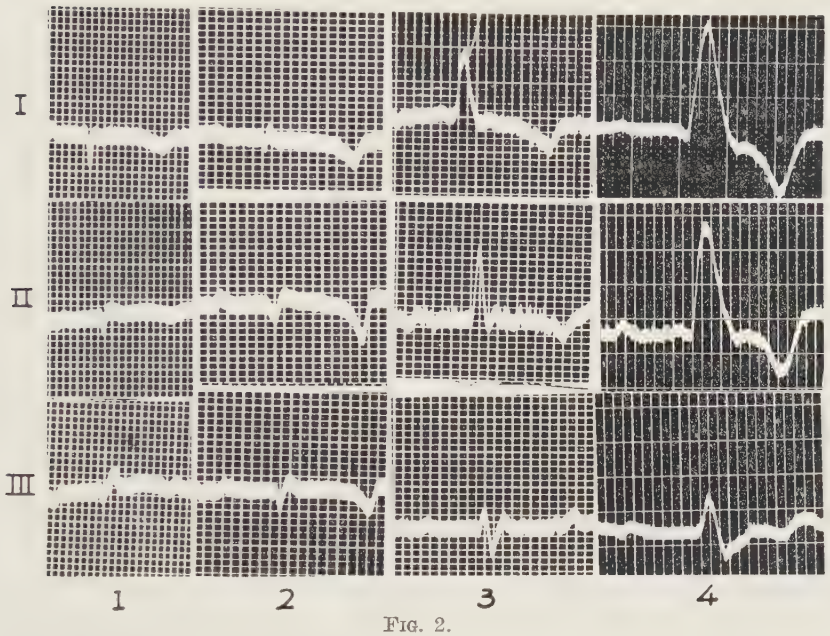

FIG. 2.

Electrocardiogram changes typical of the experimental calves: 1. Control tracing. 2. Tracing at 460 days of age. 3. Electrocardiogram at 492 days. 4. Electrocardiogram taken at 750 days of age.

ascribed to bundle branch block. A preliminary examination of various sections of the Purkinje system of right and left ventricles revealed pathological changes which are not restricted to any one particular region of the heart. A later report will describe these changes in further detail.

Arrhythmias appeared at irregular intervals both during the experimental period and during the preëxperimental period. They were perhaps more frequent during the former than during the latter but the difference was not great enough to be of certain significance.

This work is part of a project in which the Department of Dairy Husbandry and Experiment Station Chemistry is coöperating. The assistance of the staff members of these two departments, Dr. C. F. Huffman, Mr. C. W. Duncan, Dr. L. H. Greathouse, and Miss L. I. Butler, is gratefully acknowledged. The criticisms and suggestions of Dr. F. N. Wilson, University Hospital, Ann Arbor, Michigan, during the preparation of the manuscript are also gratefully acknowledged.

11270

Studies on Bovine Electrocardiogram. II. Bundle Branch Block.*

Bernard V. Alfredson and Joseph F. Sykes. (Introduced by F. N. Wilson.)

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Recent work by Cardwell and Abramson[1] and Abramson and Margolin[2] has shown that, in some species at least, the arborizations of the Purkinje network of the heart are not confined to the subendocardium as is generally assumed. By means of an injection technic they demonstrated that in the ox heart branches of the Purkinje network pierced the ventricular septum and penetrated the outer ventricular walls nearly, if not quite, to the epicardium. The branches which pierced the septum made connections with the Purkinje network of the other side. Histological studies disclosed Purkinje tissue in all muscle layers of the ventricular septum and outer ventricular walls of the hearts of dogs, sheep and pigs, but in these animals the differentiation between the Purkinje tissue and the ordinary muscle was less pronounced than in the ox heart. For this reason and because the injection method failed in these animals, the distribution of the Purkinje tissue could not be worked out as completely in them as in the ox. Electrocardiographic observations by one of us[3] suggest that contrary to the conclusions of the authors quoted,[1, 2] there may be major differences between the intraventricular conducting system of the ox heart and that of the canine heart.

In comparison with the canine and with the human heart, the ox heart is very much heavier and its walls very much thicker. It would be expected, therefore, that the QRS interval of the ox would be much longer than that of the dog or of man. In a study of the normal electrocardiogram of dairy cattle it was found that this was by no means the case. In a group of 97 cattle the mean duration of QRS was 0.09 sec with a minimum of 0.06 sec and a maximum of 0.12 sec.[3] These findings suggested that it would be worthwhile to determine the effect of section of the bundle branches of the ox heart upon the bovine electrocardiogram. Since it is difficult to carry out experi-

* Journal article No. 423 (n.s.) from the Michigan Agricultural Experiment Station.

1 Cardwell, J. C., and Abramson, D. I., *Am. J. Anat.*, 1931, **49**, 769.

2 Abramson, D. I., and Margolin, S., *Am. J. Anat.*, 1936, **70**, 250.

3 Alfredson, B. V., unpublished data.

ments of this kind in mature cattle, we have used young calves. For comparison, a number of experiments have been performed on dogs.

Procedure. In all instances sodium pentobarbital[4] was administered intravenously until surgical anesthesia supervened. The animals were held in the dorsal recumbent position. The thorax was opened and held as widely open as possible throughout the experiment by means of hook spreaders. In some of the earlier experiments the slit pericardium was stitched to the edges of the thorax, thus forming a cradle for the heart.† This procedure interfered with the manipulations necessary for efficient sectioning of branches of the His-bundle and was abandoned in favor of simply replacing the heart in the slit pericardial sac within the opened thorax. Section of the right or left bundle branch was carried out by the method described by Lewis.[5] Electrocardiograms were obtained before and after section. In 6 instances the presence of complete bundle branch block was proved by the onset of complete A-V block following a cut on the opposite side of the septum later shown to have transected the remaining bundle branch. In all cases the heart was removed at the end of the experiment, opened according to the method of Cardwell and Abramson[1] and examined to determine the location of the cuts. In view of the very clear demarcation of the right and left bundle branches in the ox heart, it is easy to determine in this way whether the bundle branches have been completely divided. Bundle branch block was successfully produced in 14 calves and in 10 dogs. Measurements of the QRS interval were made in lead II throughout, as this is generally the most satisfactory lead in cattle.[3]

Results and Discussion. The changes in the duration of QRS after section of the right or left bundle branch are recorded in Table I.

The average increase in duration of QRS on section of the right bundle branch (8 calves) was 0.013 sec, and the average increase after section of the left bundle branch (6 calves) was 0.005 sec. Right bundle branch block in dogs increased the duration of QRS 0.021 sec. Left bundle branch block increased the duration of QRS 0.029 sec. These are average values. The change in the duration of QRS in calves as compared to the change produced in dogs was, therefore, relatively insignificant.

Fig. 1 shows changes in the form of QRS typical of those produced by section of right and left bundle branches in calves and in

[4] Hafkesbring, R., and MacCalmont, W., *J. Pharm. and Exp. Ther.*, 1938, **64**, 43.

† In the first 8 bovine subjects, only 2 of which (B4 and B7) were included in the data.

[5] Lewis, T., *Phil. Trans. Roy. Soc.*, 1916, **207**B, 254, 267.

TABLE I.

Duration of QRS in Lead II Before and After Section of Right or Left Branches of the His-Bundle.

Right Bundle Branch Block					Left Bundle Branch Block				
		Duration of QRS (sec)					Duration of QRS (sec)		
Animal No.	Age (days)	Before section	After section	Increase	Animal No.	Age (days)	Before section	After section	Increase
				Calves.					
B 7	14	.05	.06	.01	B 4	7	.04	.05	.01
B 9	21	.05	.06	.01	B19	44	.04	.05	.01
B10	21	.05	.06	.01	B20	3	.05	.05	
B12	14	.055	.07	.015	B21	28	.05	.05	
B13	14	.055	.07	.015	B22†	28	.055	.065	.01
B15	14	.055	.08	.025?*	B23	3	.05	.05	
B17	240	.06	.07	.01					
B18	46	.05	.06	.01					
Mean		.053	.066	.013			.047	.052	.005
				Dogs					
D 5		.08	.10	.02	D 2		.05	.08	.03
D 7		.05	.08	.03	D11		.055	.08	.025
D 8		.04	.07	.03	D12		.05	.08	.03
D13		.055	.065	.01					
D14		.065	.08	.015					
D15		.05	.08	.03					
D16		.055	.07	.015					
Mean		.056	.077	.021			.051	.08	.029

* The exact QRS interval was difficult to determine in this instance because the end of the QRS group was ill defined.

† Failure to record lead II in the control tracing due to insufficient opening of the camera shutter necessitated the taking of data from lead III in this subject.

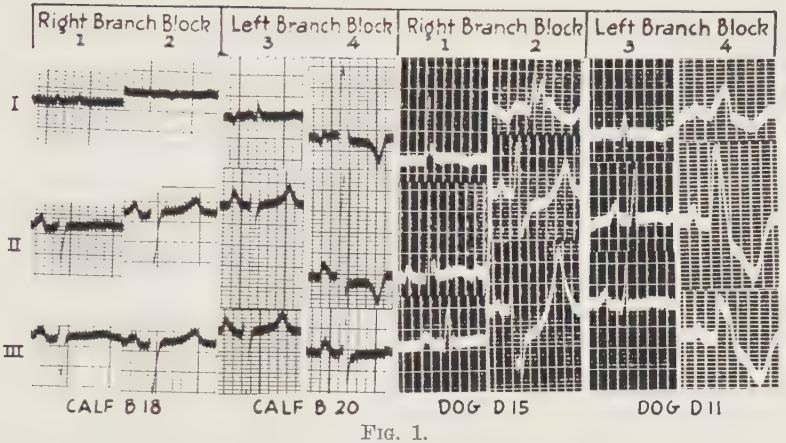

FIG. 1.

Electrocardiographic changes typical of those produced by section of the right and left bundle branches in calves and dogs. No. 1 and 3 are control records; No. 2 and 4 are records taken after section of the right or left bundle branches.

dogs. The canine left and right branch block curves are similar in form to those described by Lewis[5] and are very different from the control curves. The right branch block curves of the calves, however, were scarcely distinguishable from the control curves in the majority of instances. In some instances there was a slight increase in the size of S and in the voltage of T in leads II and III.

In comparison with the controls the left branch block curves of the calves showed much greater changes in the form of the ventricular complex but the character and magnitude of these changes varied greatly in different experiments. The form of the control curves was quite constant.‡ The deflections of Lead I were extremely small. In the other leads QRS consisted of an upright deflection (R) followed by a downward deflection (S). The relative size of R and S was variable but in most instances the latter was larger than the former. The T-waves were usually upright but of small voltage. After section of the left bundle branch the voltage of the largest QRS deflection was greater than in the control. In one instance QRS was represented by a large downward deflection in Lead I and a large upward deflection in Leads II and III. In this instance T_2 and T_3 were large and inverted. In 2 instances large R-waves and large inverted T-waves appeared in Leads I and II. In another experiment the changes were similar to those just described but less pronounced; in another the R deflection became larger and the T-wave became inverted in Leads II and III but the deflection in Lead I remained

‡ This uniformity is not seen in electrocardiograms of the normal bovine subject when taken in the standing position.[3]

small. In the remaining experiments the changes were very slight. Distinct notching of the larger QRS deflections did not occur in any experiment.

Compared to the electrocardiographic changes produced by right and by left bundle branch block in man and in the dog, those produced by cutting either of the bundle branches of the calf's heart are extremely small and as regards the form of the electrocardiogram conspicuously variable. This difference suggests that there is a decided difference in the distribution of the intraventricular conducting system between man and the dog, on the one hand, and the calf on the other. The smallness of the QRS interval of the bovine electrocardiogram points in the same direction. It is difficult to understand how the cardiac impulse can spread so quickly over the ventricular muscle of so large a heart if it spreads with approximately the same speed and in the same manner as in the human and the canine heart. The pronounced increase in the QRS interval produced by bundle branch block in man and in the dog has been attributed to the slow spread of the cardiac impulse through the ordinary muscle of the ventricular septum. The absence of a similar increase in the QRS following section of the bundle branches of the calf's heart suggests that in this animal the Purkinje networks of the 2 ventricles are connected by strands of specialized tissue which pierce the ventricular septum. Penetration of the outer ventricular walls by Purkinje tissue would for similar reasons account for the smallness of the QRS interval of the bovine electrocardiogram in comparison with the size of the ox heart. The differences between the branch block curves of the dog and those of the calf suggest that contrary to the conclusions of Abramson and his fellow workers the distribution of the Purkinje tissue is not similar in the two species, and that this tissue does not penetrate the ventricular septum of the canine heart. The same conclusion may be drawn with respect to the human heart and the hearts of other species in which bundle branch block induces electrocardiographic changes of comparable magnitude.

Summary. Changes in the duration and form of QRS after section of the branches of the His-bundle are much less pronounced in calves than in dogs. The difference between these two species is attributed to differences in the distribution of the intraventricular conducting system.

The authors wish to express their thanks for the assistance and advice of Dr. F. N. Wilson, University Hospital, Ann Arbor, Michigan, and Dr. E. T. Hallman, Michigan State College, East Lansing. The experimental animals were generously donated by the Dairy Department of Michigan State College.

11271

Fermentation of Pyruvic Acid by *Clostridium botulinum.**

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It has been reported[1] that the fermentation of glucose by Types A and B *Clostridium botulinum* differs from the majority of bacterial fermentations in that ethyl alcohol and carbon dioxide are the main products of the fermentation, only small amounts of acetic and lactic acids together with traces of hydrogen being formed. This study has been extended to include the fermentation of pyruvic acid, which may be an intermediate compound in the fermentation of glucose and also in the degradation of amino acids such as alanine, by *Cl. botulinum.*[2]

The experiments reported below were carried out with washed suspensions of Type A (E-43) *Cl. botulinum* although studies with Type B gave essentially the same results. Glucose-broth (800 ml) containing 0.1% Difco yeast extract was inoculated with 1.0 ml of a beef-brain culture of the organism and incubated for 20 hours at 37°C in a McIntosh and Fildes anaërobic jar. The culture was then centrifuged and the cells suspended in distilled water. The suspension was diluted with an equal volume of M/7.5 phosphate buffer and placed in the central chamber of Warburg vessels. A rapid stream of O_2-free H_2 was passed through the vessels for 10 minutes and they were then equilibrated 10 minutes before tipping in the sodium pyruvate from the side-arm. CO_2 production was determined at 37°C by the Warburg technic, the initial and final bound CO_2 being determined following the addition of 10% sulfuric acid to the contents of separate Warburg vessels. In the semi-macro experiments, Warburg vessels of 40 ml capacity were employed with Clerici fluid in the manometers.

It was observed that pyruvic acid is rapidly decarboxylated by washed suspensions of *Cl. botulinum,* Q_{CO_2} values of 25-30 being observed under optimal conditions. Typical results over a pH range of 5.6 to 7.5 are reported in Fig. 1. Following correction of the results for CO_2 bound by the phosphate buffer it is apparent that the pH optimum lies near 6.0. Accordingly the majority of the fermen-

* Aided in part by a grant from the Rockefeller Fluid Research Fund.

1 Clifton, C. E., *J. Bact.*, 1940, in press.

2 Clifton, C. E., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 338.

tations were carried out at this pH although similar results were obtained at pH 7.0. The fermentation of glucose differs from that of pyruvic acid in that the rate is much less dependent on the pH over a range of 5.6 to 7.8.

In preliminary experiments ethyl alcohol and acetic acid were identified as the main non-gaseous products of the fermentation. Alcohol was determined by oxidation with bichromate following distillation from alkaline solution and volatile acids by steam-distillation of the acidified residue. Ethyl alcohol was identified by the iodoform test and by the fact that acetic acid appears to be the only acid produced on oxidation with bichromate. This acid and that produced in the fermentation was identified as acetic by Duclaux distillations of the combined steam-distillates from several large-scale experiments. No gas other than CO_2 was produced and the CO_2 liberated by control suspensions was negligible. In general about 5% of the pyruvic acid was reduced to lactic acid during the course of the fermentation.

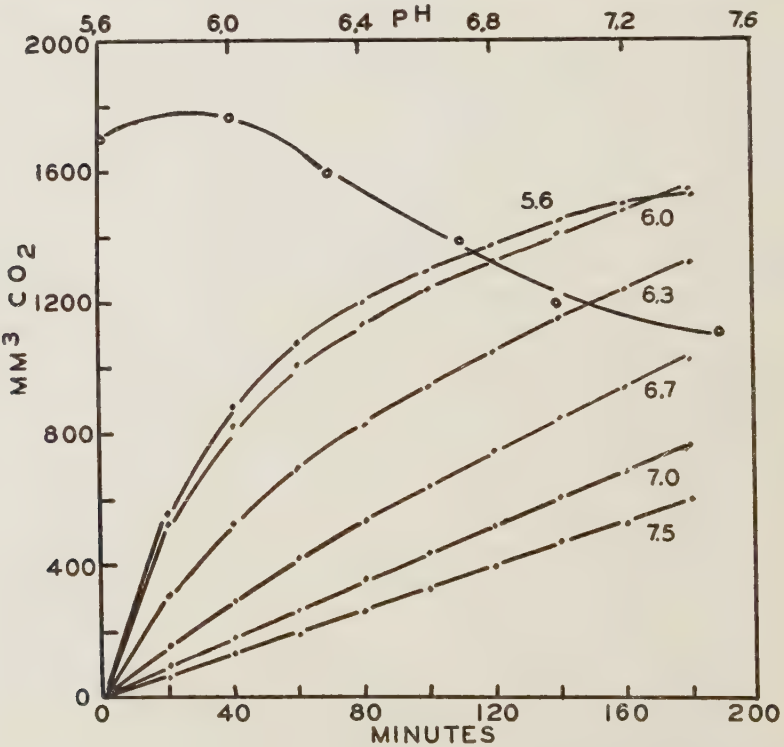

FIG. 1.

Influence of pH on the rate of CO_2 liberation during the fermentation of pyruvic acid by *Cl. botulinum*. O——O Total CO_2 produced, corrected for bound CO_2, from 0.2 ml M/2 Na pyruvate in 180 minutes.

TABLE I.

Pyruvate fermented	mg 44	Mols/ mol pyruvate	mg 44	Mols/ mol pyruvate
Products				
CO_2	19.4	.88	19.8	.90
CH_3CH_2OH	12.3	.53	13.0	.56
CH_3COOH	13.2	.44	13.8	.46
Total	44.9		46.6	

The results of typical duplicate semi-macro experiments at pH 6.0 are presented in Table I.

Similar results were obtained when the fermentation was carried out in an atmosphere of O_2-free N_2.

The results suggest that pyruvic acid is decarboxylated and the acetaldehyde produced dismuted with the production of equimolar quantities of ethyl alcohol and acetic acid. The above results are in fair agreement with this hypothesis since no correction was made for the small amounts produced by the organisms alone, the quantities being too small for accurate determination. In general the amounts of CO_2 recovered were somewhat less than the theoretical while the ethyl alcohol tended to run somewhat high. This tendency was also observed in macro experiments in which CO_2 production was determined by absorption of the liberated gas in standard $Ba(OH)_2$ solution. Possibly a small amount of CO_2 is utilized during the fermentation. It appears, therefore, that the fermentation of pyruvic acid may be represented as

$$2\ CH_3COCOOH + H_2O = 2\ CO_2 + CH_3CH_2OH + CH_3COOH$$

If the fermentation of glucose proceeds by way of pyruvic acid, dismutation of acetaldehyde may account for the small amount of acetic acid produced, the bulk of the acetaldehyde being reduced to alcohol by a H-donor other than another molecule of acetaldehyde.

11272

Utilization of Serine by *Clostridium botulinum.*[*]

C. E. Clifton.

From the Department of Bacteriology and Experimental Pathology, Stanford University, California.

A number of amino acids are utilized by washed suspensions of *Clostridium botulinum,* for the most part in coupled reactions between pairs of different amino acids.[1, 2] For example, alanine is deaminatively oxidized to CO_2 and acetic acid by glycine or proline which are reduced to acetic acid or δamino-n-valeric acid, respectively. However, amino acids such as leucine and particularly serine appeared to be deaminated when present singly in washed suspensions of this organism. The utilization of serine has been studied in detail by the technic already described,[3] ammonia being determined by the method of Parnas as described by Niederl and Niederl.[4]

Serine is decarboxylated in the presence of washed suspensions of Types A or B *Cl. botulinum* at a rate (Q_{CO_2} of 7-10) approximately one-third of that observed with pyruvic acid (Q_{CO_2} of 25-30) as the substrate at optimal pH values for both reactions. Typical results of CO_2 production are presented in Fig. 1.

It is apparent that the pH optimum for the utilization of serine by *Cl. botulinum* lies on the alkaline side of neutrality, probably near pH 7.5. This is in marked contrast with the pH optimum of 6.0 observed with pyruvic acid as the substrate. In further studies it was observed that apparently only one optical form of serine is attacked at an appreciable rate, deamination and decarboxylation of *dl*-serine proceeding only approximately 50% to completion.

In semi-macro experiments in Warburg vessels of 40 ml capacity with Clerici fluid in the manometers the fermentation was studied in more detail. Ammonia, CO_2, ethyl alcohol and acetic acid were identified as the chief products of degradation of serine. In these experiments the reaction was not allowed to go to completion due to the slow rate observed by the time approximately one-half of the serine had been utilized. Typical results on the utilization of serine

* Aided in part by a grant from the Rockefeller Fluid Research Fund.

1 Clifton, C. E., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 338.

2 Clifton, C. E., *J. Bact.*, 1940, in press.

3 Clifton, C. E., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 585.

4 Niederl, J. B., and Niederl, V., *Micromethods of Quantitative Organic Elementary Analysis,* 1938, 51-59, John Wiley and Sons, New York.

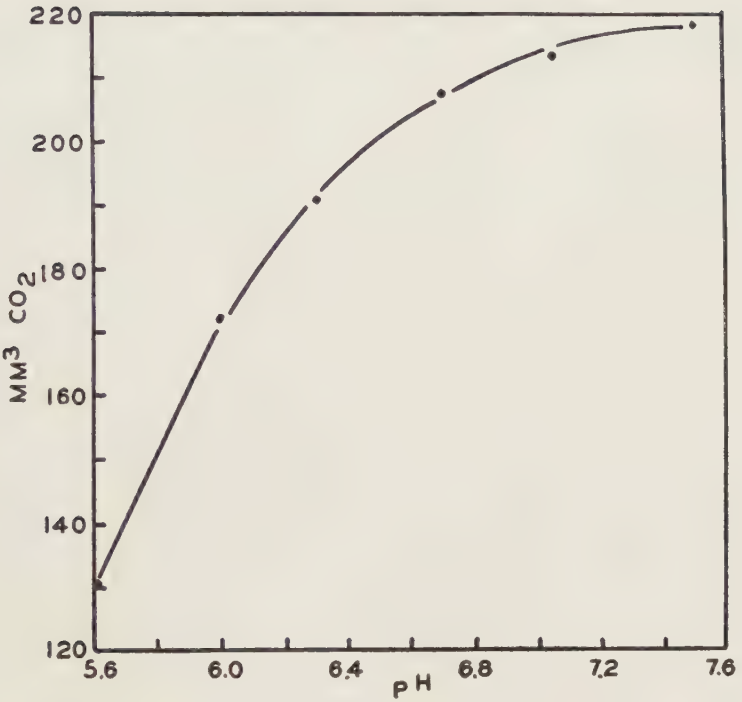

FIG. 1.

Influence of pH on CO_2 production, corrected for bound CO_2, by *Cl. botulinum* from 0.2 ml M/10 *dl*-serine in 90 minutes at 37°C.

by washed suspensions of Type A *Cl. botulinum* in M/15 phosphate buffer of pH 7.2 are recorded in Table I, the fermentation being carried out in an atmosphere of O_2-free H_2. Similar results were obtained in an atmosphere of O_2-free N_2 and with washed suspensions of Type B *Cl. botulinum*. The quantity of serine fermented is based on the NH_3 liberated during the course of the experiment.

The results suggest that serine is deaminated and rearranges to form pyruvic acid which is then fermented, or, that serine is de-

TABLE I.

	mg	Mols/mol serine	mg	Mols/mol serine
Initial serine	52.5		52.5	
Final serine	28.0		32.5	
Serine utilized	24.5		20.0	
Products:				
NH_3	3.98	1.00	3.25	1.00
CO_2	8.98	0.87	7.17	0.86
CH_3CH_2OH	6.15	0.55	5.06	0.57
CH_3COOH	7.20	0.51	6.00	0.53
Total	26.31		21.48	

carboxylated and deaminated at the same time to yield acetaldehyde which is dismuted to form equimolar quantities of ethyl alcohol and acetic acid. If the reaction proceeds by way of pyruvic acid, deamination appears to be the controlling factor of the rate of utilization of serine as evidenced by the marked shift in pH optimum from 6.0 for pyruvic acid to the neighborhood of 7.5 for serine. These results, considering the experimental errors involved, suggest that the degradation of serine by washed suspensions of *Cl. botulinum* may be represented as

$$2\ CH_2OH\ .\ CHNH_2COOH + H_2O = 2\ NH_3 + 2\ CO_2 + CH_3CH_2OH + CH_3COOH$$

The closely related amino acid, alanine, is not attacked directly by *Cl. botulinum* but only when a suitable H-acceptor is also present. Therefore, substitution of an hydroxyl group for a H-atom on the β-carbon of alanine produces a compound that may be employed singly as a source of energy by *Cl. botulinum*. These above results show that *Cl. botulinum* may obtain a portion of its energy requirements by direct utilization of amino acids such as serine as well as through coupled reactions between pairs of different amino acids (Stickland reaction) as previously described.

11273 P

Nasopharyngeal Cultures in Pertussis.*

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The generally accepted method for the bacteriological diagnosis of pertussis is the cough-plate culture, originally described by Chievitz and Meyer,[1] or some modification of their procedure. The percentage of positive cultures obtained by this technic varies considerably in the hands of different workers and under various conditions.[2]

We have found the cough-plate method satisfactory for older children, but in infants we have obtained positive cultures in only

* Supported by a grant from the Committee on Therapeutic Research of the American Medical Association and the Fluid Research Fund of the University of Rochester School of Medicine and Dentistry.

1 Chievitz, J., and Meyer, A., *Ann. de l'Inst. Pasteur*, 1916, **30**, 503.

2 Sauer, L. W., *J. Ped.*, 1934, **5**, 246.

about 25%, even when the cultures were made during the catarrhal period of the disease. This low incidence seems to be due to the fact that the young infant does not cough as vigorously as does the older child, thereby resulting in an inadequate inoculation of the medium.

During the course of a comparative study of various technics for making cultures, we were impressed by the favorable results obtained from culturing the nasopharynx. Recent reports[3, 4, 5] have described the advantages of the nasopharyngeal culture in the bacteriological diagnosis of pneumonia. This present report describes the results obtained by making simultaneous nasopharyngeal, throat and cough-plate cultures in a series of 25 consecutive cases of pertussis observed in the pediatric clinic during the past 4 months. The procedure follows.

The medium was prepared as previously described[6] and poured into ordinary Petri dishes. Medium more than 3 days old was discarded. The cough-plate cultures were made by exposing the medium during a paroxysm at a point about 6 inches in front of the patient's mouth. The nasopharyngeal cultures were taken by passing a sterile swab, consisting of a small bit of cotton tightly wrapped about the end of a thin, flexible copper wire, through the nose until it touched the posterior wall of the pharynx. Throat cultures were taken by means of a similar swab.

The results obtained in the present comparative study are shown in Table I.

Cultures were made on 25 cases, 17 of which were but 2 years of age or less. In this group, it is apparent that the results obtained by nasopharyngeal culture were distinctly superior to those by the throat culture or by the cough-plate method. In the 25 primary cultures, 17 were positive. Of this group, the nasal culture was positive in 14 instances, as compared to 8 positive cough-plates and to 4 positive throat cultures. Of the entire group of 40 cultures, there were 22 positive nasal, 10 positive cough-plates and 5 positive throat cultures. In only 3 cases were the cough-plate cultures positive when negative nasopharyngeal cultures were obtained. These patients were 4, 6, and 7 years of age, respectively, and were presumably able to cough vigorously.

3 Hodes, H. L., Stifler, W. C., Jr., Walker, E., McCarty, M., and Shirley, R. G., *J. Ped.*, 1939, **14**, 417.

4 Poole, F. D., and Fousek, M. D., *J. A. M. A.*, 1939, **113**, 1854.

5 Auger, W. J., *J. Ped.*, 1939, **15**, 640.

6 Bradford, W. L., and Slavin, B., *J. Clin. Investigation*, 1937, **16**, 825.

TABLE I.
A Comparison of Culture Methods in Pertussis.

Patient	Age	Wk of disease	Cough-plate	Throat	Naso-pharynx	Patient	Age	Wk of disease	Cough-plate	Throat	Naso-pharynx	Patient	Age	Wk of disease	Cough-plate	Throat	Naso-pharynx
We	8 wk	2	0	0	+	Be	2 yr	2	0	0	+	Bi	9 mo	1	0	0	0
		4	0	0	+			4	0	0	0			2	+	0	++
		6	0	0	0			8	0	0	0			4	0	0	0
Ho	3 mo	4	+	+	+	Wa	6 wk	2	0	0	0	Sa	5 mo	3	0	0	0 +
		6	0	0	+			3	0	0	0	O'K	3 yr	2	0	0	
Al	1 yr	5	0	0	+			4	0	0	+	O'K	6 ''	2	+	0	0
		6	0	0	+	Sp	3 mo	5	0	0	0	Le	6 ''	4	0	0	0
DeL	3 mo	5	0	0	0			7	+	+	+	Co	4 ''	5	+	0	0
		6	0	0	0			10	0	0	0			8	0	0	0
Ce	2 yr	1	0	0	+	McK	2 yr	2	0	0	+	Le	7 ''	4	+	0	0
Bu	2 ''	4	+	0	+	Bo	2 ''	3	0	+	+	Le	8 ''	2	0	0	+
		6	0	0	+	Ko	4 mo	2	0	0	+	Hn	4 ''	4	0	0	0 +
Sm	7 wk	3	0	0	0	Hu	2 yr	1	+	+	+	Ba	8 mo	1	+	+	
Ar	3 yr	2	+	0	+												

Of a total of 25 positive cultures, there were 22 nasal, 5 throat, and 10 cough-plate.
Of the 25 primary cultures, 17 were positive; 14 nasal, 4 throat and 8 cough-plate.

In addition to the higher percentage of positive nasopharyngeal cultures obtained, the difference in the number of colonies which developed on the plates was striking. In repeated instances, there were only a few colonies of *Hemophilus pertussis* on a cough-plate or throat-plate culture, while the nasal culture revealed numerous colonies. Sometimes the growth was practically pure. The nasal culture often showed but a minimal growth of secondary invading organisms, while a heavy growth, on the other hand, usually occurred on the media inoculated from the throat. We believe that this difference in the occurrence of secondary invading organisms largely explains the difference in the percentage of positive cultures of *Hemophilus pertussis* isolated from the two sources. It may be, however, that the pertussis bacillus occurs in greater numbers in the nasopharynx during the disease and remains there for a longer period of time during convalescence. Further study will be necessary to decide this point.

A comparative study of the cough-plate, throat and nasopharyngeal cultures in pertussis indicates that the nasopharyngeal method is definitely the best, particularly for use in infants.

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Anticatalase Activity of Sulfanilamide and Related Compounds. V. Bacteriostatic Activity of some Sulfonhydroxamides.

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This is a report of a study in which the ability of sulfanilamide to produce catalase inhibition is compared with that exhibited by acyl aminobenzenesulfonhydroxamides. Since the latter compounds contain a hydroxylamino group, they are analogous in structure to the intermediate assumed to be responsible for the anti-catalase activity of sulfanilamide in bacterial cultures.[1, 2, 3] Possibly because of the presence of this group, the sulfonhydroxamides are capable of exerting a more rapid and more intense bacteriostatic effect than sulfanilamide.

1 Locke, A., Main, E. R., and Mellon, R. R., *Science*, 1938, **88**, 620.

2 Main, E. R., Shinn, L. E., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 272.

3 Shinn, L. E., Main, E. R., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 591.

Bacteriostatic Action. Fig. 1 compares the speed and intensity of the bacteriostatic action of sulfanilamide and caproylaminobenzenesulfonhydroxamide, the latter being chosen as typically representative of the group. Cultures, made by inoculating 10 cc quantities of veal infusion broth, contained in 50 cc Erlenmeyer flasks, with 0.0001 cc of a highly virulent, 18-hr broth culture of Type I pneumococcus, were incubated at 39.5°C. After 1.5 hrs, plate counts were made and the compounds added as freshly prepared solutions in 60% alcohol, the concentrations of which were so adjusted that an addition of 0.1 cc would give the desired final concentrations. The same volume of 60% alcohol was added to control cultures. Incubation was continued and samples were withdrawn for plate counts 0.75, 1.75, and 3.5 hrs after addition of the drugs.

The rate of generation was estimated from the counts by the formula:

$$\frac{\text{count at the end of the observed interval}}{\text{count at the beginning of the interval}} = 2^n$$

The degree of retardation in rate of multiplication was obtained as the difference between the rate of generation in the control culture and in the culture containing the added drugs.

Example:

Count 1.75 hrs after addition of compound = 1220
Count 3.5 hrs later = 6100

$\frac{6100}{1220} = 5.0$ and therefore represents a 5.0-fold increase in population.

From the equation, $5.0 = 2^n$, $n = 2.3$ (total number of generations produced).

Number of generations produced per hr $= 2.3/1.75 = 1.3$.

Since the number of generations per hr in control cultures for the corresponding time interval was 1.8, the difference, 0.5, represents the inhibition in rate of multiplication.

The results show a distinct difference in rate and intensity of bacteriostatic action of the two compounds. In the cultures containing sulfanilamide (shaded columns, Fig. 1), a slight stimulation in rate of multiplication was observed at the end of the first interval. This was followed by inhibition which continued to increase during the remainder of the observation period. Inhibition by the sulfonhydroxamide (solid columns) was detectable by the end of the first interval and rapidly increased in magnitude, presumably as a result of increasing peroxide accumulation. By the end of the second period of observation, the inhibition was 2.5 times greater than that of sulfanilamide although the concentration was less than one-third as great.

Peroxide Accumulation and Growth Inhibition. Table I indicates

the comparative capacities of sulfanilamide and the sulfonhydroxamides for producing peroxide accumulation and growth inhibition. The ratios of peroxide concentration to amount of growth were determined under the experimental conditions previously described.[4] Alcoholic solutions of the drugs were used as in the experiments described above, but were added before inoculation. The inoculum, 0.1 cc of an 18-hr culture, was larger than that used in the above experiments in order that growth would be sufficiently large to be measured turbidimetrically from the fourth to the seventh hour of growth. Only the ratios found at the seventh hour are reported in the table. Under the conditions of the experiment, peroxide was usually detectable by the peroxidase test* by the third hour.

In cultures containing 0.00014 to 0.00020 *M* caproylaminobenzenesulfonhydroxamide, the peroxide concentration per unit of growth was from 2 to 4 times greater than in control cultures. With a 0.00052 *M* concentration there was no visible growth during the 7 hours. In cultures containing 0.00052 *M* sulfanilamide, peroxide concentration per unit of growth was, on the average, 4 times greater than that of the control. In the presence of 0.00052 *M* acetamidobenzenesulfonhydroxamide the ratio was from 2 to 3 times greater and therefore this compound was only slightly less effective than sulfanilamide in the same concentration. Toluene- and benzenesulfonhydroxamides were approximately equal in effect to the acetamido compound.

Anti-Catalase Activity. The hydroxamides listed in Table I showed anti-catalase activity equal to or greater than that of irradiated sulfanilamide. The activity was not, however, constant. Upon standing in solution the sulfonhydroxamides undergo a change, shown by the development of a yellow color and an altered reaction with *o*-tolidine.

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[4] Main, E. R., Shinn, L. E., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 115.

* A slight modification of the method previously described (Main and Shinn, *J. Biol. Chem.*, 1939, **128**, 417) was used. An aqueous extract of dried horseradish was used in place of the potato extract as the source of peroxidase, as suggested by Fuller and Maxted (*Brit. J. Exp. Path.*, 1939, **20**, 177). Determinations can be made over the range, 2 to 20γ per cc, while with potato extract, the range is 6 to 30γ.

It was necessary to use an additional blank in the determination of peroxide in the presence of sulfonhydroxamides, since, after standing at a pH of 7.0 or above for one-half hour or more, solutions of the sulfonhydroxamides give a yellow to orange color upon addition of the *o*-tolidine reagent. To make color comparisons with the peroxide standards, a blank consisting of sterile broth which contains the same concentration of compound as the culture is placed back of the standard tube in the comparator.

TABLE I.

Effect of Sulfonhydroxamides on Growth and Peroxide Accumulation in Broth Cultures of Type I Pneumococcus.

Ten cc quantities of veal infusion broth containing 0.2% glucose and the compounds in the concentrations given below were inoculated with 0.1 cc of an 18-hr broth culture of Type I pneumococcus. Samples were removed at intervals for determination of growth and peroxide content. The ratios, A and B, were calculated from determinations made after 7 hr growth.

Compound added	Millimols per l†	H_2O_2 concentration per unit of growth‡ in A Control cultures	H_2O_2 concentration per unit of growth‡ in B Cultures contg. compds.	Ratio, B : A
4-*n*-Caproylaminobenzenesulfonhydroxamide*	0.52	0.3	—§	—
	.20	.3	1.1	3.7
	.20	.4	.8	2.0
	.17	.4	.8	2.0
	.14	.2	.5	2.5
	.14	.4	.7	1.8
4-Acetamidobenzenesulfonhydroxamide	.52	.2	.6	3.0
	.52	.2	.4	2.0
	.52	.3	1.0	3.3
p-Toluenesulfonhydroxamide	.52	.2	.5	2.5
	.52	.2	.5	2.5
	.52	.3	.4	1.3
Benzenesulfonhydroxamide	.52	.2	.6	3.0
	.52	.3	.7	2.3
	.20	.3	.7	2.3
	.17	.4	.5	1.3
Sulfanilamide	.52	.3	1.2‖	4.0

* The sulfonhydroxamides were furnished to us through the courtesy of Sharp and Dohme, Inc.

† Stock solutions of the compounds in 60% alcohol were prepared in such concentrations that 0.1 cc contained the amount needed to give the final concentration. The control cultures contained 0.1 cc alcohol.

‡ Growth was estimated by comparisons with $BaSO_4$ standards which had been calibrated against a culture arbitrarily selected to represent 100% growth. The growth units are therefore percentages relative to the standard culture. H_2O_2 concentration is expressed in micrograms per cc.

§ There was no visible growth in cultures containing this concentration.

‖ This figure represents the average of all determinations made with sulfanilamide-containing cultures, which were included regularly as controls.

Discussion. Sulfonamide derivatives studied heretofore have contained a para amino group which is convertible into a hydroxylamino group, giving the compound anti-catalase properties. In bacterial cultures, these compounds check the action of catalase and thereby cause increased accumulation of peroxide. The amount of active intermediate present may be very small, its effect depending on its continuous release in the vicinity of the cell. A compound which contains a preformed hydroxylamino group should act more rapidly

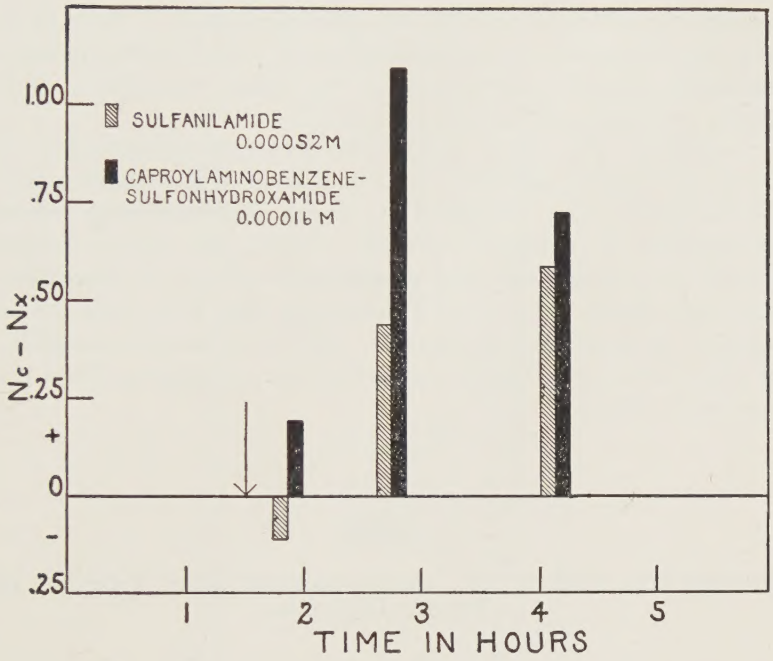

FIG. 1.

Comparison of the rates of development of bacteriostasis in the presence of sulfanilamide and 4-*n*-caproylaminobenzenesulfonhydroxamide.

N_c is the number of generations produced per hour in the control cultures and N_x the number produced in the cultures containing compound added. The additions were made at the time indicated by the arrow. Each column represents the average of results from 10 experiments.

and, because of the presence of active substance in high concentration, should have a more intense effect. The experiments reported here indicate that this is the case with the acyl aminobenzenesulfonhydroxamides, compounds of the structure, $RNH\text{-}C_6H_4\text{-}SO_2NHOH$, where R is the caproyl, valeryl, heptanoyl or acetyl group. When compared mole for mole, caproylaminobenzenesulfonhydroxamide is a much stronger bacteriostatic agent than sulfanilamide. Bacteriostatic activity is demonstrable almost immediately and increases rapidly while peroxide is accumulating. Since the toluene and benzenesulfonhydroxamides, compounds which contain no para amino group, caused marked stasis and peroxide accumulation, the activity of the acyl compounds *in vitro* is apparently not due to a deacylated amino group.

The results given here apply only to stasis in broth cultures. The concentration of the hydroxamide required to produce the same amount of stasis in blood culture is appreciably greater. Results of *in*

vivo experimentation[5] indicate that when given daily in doses of 1 mg per g for 6 days, the acyl aminobenzenesulfonhydroxamides and sulfanilamide possess approximately the same therapeutic activity against pneumococcus infection in mice.

Summary. The acyl aminobenzenesulfonhydroxamides have strong anti-catalase activity and, when present in broth cultures of the pneumococcus, cause inhibition of growth associated with increased accumulation of hydrogen peroxide. When the caproyl compound is added to growing cultures, inhibition of growth is detectable almost immediately but reaches a maximum only after time for accumulation of peroxide has elapsed. Inhibition by sulfanilamide, on the other hand, is detectable somewhat later and approaches a maximum more slowly.

11275

Gonadotropic Potency of Gonadectomized Rats' Pituitary after Tryptic Digestion.

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The evaluation of the amount of the follicle-stimulating and luteinizing hormones of the pituitary of normal as well as gonadectomized animals has interested a number of investigators. The recent reports of McShan and Meyer[1] and Chen and van Dyke[2] on tryptic digestion of pituitary extract appear to afford a convenient means of estimating the relative amounts of these fractions, as the luteinizing activity is largely destroyed by trypsin to which the follicle-stimulating activity is resistant. In the following experiments the gonadotropic activity of castrate rats' pituitary following tryptic digestion was compared with that of the castrates' pituitary not subjected to such treatment.

The donors of the pituitary consisted of 24 male and 38 female albino rats which were gonadectomized at the age of 1-3 months. Three to 6 months (usually 3 months) after gonadectomy, the animals were sacrificed. The anterior pituitary was obtained, weighed, and ground up fresh in an appropriate quantity of 0.02%

5 Cooper, F. B., Gross, P., and Lewis, M., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 491.

1 McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1938, **126**, 361.

2 Chen, G., and van Dyke, H. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 172.

TABLE I.
Gonadotropic Potency of Male and Female Castrates' Pituitary Before and After Tryptic Digestion.

Group	Type of pituitary*	Total dose, mg	No. of immature rats	Mean and S.E. of paired ovarian wt, mg	Mean and S.E. of uterine wt, mg	Groups compared	P†	
							ovaries	uterus
1	F	5.0	9	69.76 ± 4.90	82.10 ± 3.61	1-2	.0	.17
2	F, T	10.0	12	23.73 ± 1.42	63.77 ± 10.66	1-3	.66	.88
3	M	5.0	10	66.65 ± 4.74	82.89 ± 4.45	2-4	.65	.06
4	M, T	10.0	10	22.81 ± 1.33	40.35 ± 1.67	3-4	.0	.0
5	FSH	1.0	10	52.27 ± 8.70	96.70 ± 6.68	5-6	.69	.33
6	FSH, T	1.0	11	47.55 ± 7.00	81.88 ± 9.96			

* F—Female castrate; M—Male castrate; FSH—Follicle-stimulating hormone; T—Trypsin-digested.

† Probability that random sampling would give as great a difference according to Fisher's method[3] for testing significance of difference of means.

[3] Fisher, R. A., *Statistical Methods for Research Workers,* Oliver and Boyd, Edinburgh, 1936.

Na_2CO_3 solution. To an aliquot portion of the material sufficient trypsin* was added to make 0.05%. A second portion was left without trypsin. The pH was adjusted to approximately 9.6 (thymol blue). Both portions were incubated for 2 hours at 38°C. The pH was then adjusted to about 7.6 (bromthymol blue) and 0.2 mg of merthiolate per cc was added to each solution. These were assayed in littermate 21-day female rats. In all cases the total dose was contained in 4 cc given in 8 equal subdoses over 4 days. When not in use, the solutions were kept at 4°C in the refrigerator.

The results of the experiments are summarized in Table I. Five mg of the male and the female castrates' pituitary not digested with trypsin contained clearly greater gonadotropic activity than 10 mg of the castrates' pituitary following its digestion by trypsin. It thus appeared that the amount of trypsin-digestible fraction (*i. e.*, the luteinizing activity) was considerable in both the male and female. Furthermore there appeared to be no sex difference in the gonadotropic potency of the pituitary either with or without tryptic digestion in the gonadectomized rat. Under the conditions of the experiment a sample of follicle-stimulating hormone from sheep pituitary was not significantly affected by similar treatment.

A few sections of the ovaries of Groups 1 and 3 revealed varying degrees of luteinization. The number of sections studied, however, did not permit a statement as to the relative degree of luteinization in respect to sex. With the dose level used in Groups 2 and 4, no luteinization was present in any ovaries.

Summary. The gonadotropic potency of the pituitary of spayed and castrated rats was determined in the immature female rat before and after tryptic digestion. There was a great reduction of potency of the pituitary following digestion by trypsin in both the male and female.

I am indebted to Dr. Graham Chen for suggestions in these experiments and for supplying me with a sample of follicle-stimulating hormone.

* From the Central Scientific Company of Chicago.